Genome-wide Identification of the Laccase Gene Family in White Jute (*Corchorus capsularis*): Potential Targets for Lignin Engineering in Bast Fiber

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Abstract:

The industrial bast fibre crop jute (*Corchorus* sp.) is known for its long lignocellulosic multi-utility fibres. Information on jute fibre lignification is limited, and many enzymes in the jute lignin pathway are not well documented. One such enzyme is laccase (EC 1.10.3.2), involved in the final polymerization step of lignification. A whole-genome search of white jute (*Corchorus capsularis*) revealed 34 putative laccase (*CcaLAC*) genes. Phylogenetic analysis categorized these genes into six groups, with 17 predominantly expressed in phloem tissue, 9 in leaf, and 4 in xylem and roots. A steady increase in gene expression, from plantlets to crop harvest, was observed for several *CcaLAC*s. Some were selected for further analysis based on homology with *Arabidopsis* lignin pathway-modifying laccases (*AtLAC*s). Transcriptomics data confirmed their expression in phloem tissues, with some showing significantly lower expression in *dlpf*, a low-lignin fibre-containing white jute mutant. Changes in *CcaLAC* expression were observed under abiotic stresses like ABA hormone and copper heavy metal. Target sites for Ath-miR397a and Ath-miR397b were predicted in 11 and 9 *CcaLAC*s, respectively, suggesting possible post-transcriptional modification via microRNA. Subcellular localization showed *CcaLAC*s in multiple plant cell compartments. Protein structure predictions revealed up to 10 motifs in CcaLACs, with 18 containing transmembrane helices. Overall, *CcaLAC28* and *CcaLAC32* are likely involved in the lignification process of phloem (bast) in white jute. Modifying these genes could enhance our understanding of

lignification and potentially lead to the development of low-lignin jute fibres, meeting high industrial demands globally.

Keywords: Jute; Laccase; Whole Genome Search; microRNA; Abiotic stress; Lignin pathway

Highlights

- Jute is known for its long lignocellulosic fibres.
- 34 putative laccase genes were identified in white jute.
- 17 laccase genes are mainly expressed in phloem tissue.
- *CcaLAC28* and *CcaLAC32* are key candidates for lignification.
- Laccase genes showed changes under abiotic stresses.

Key Message

Identified 34 laccase genes in white jute, key for understanding and engineering lignification in fibres.

1. Introduction:

Laccases (LAC) are a diverse group of multicopper oxidases found in multiple organisms, including higher plants (**Claus, 2004**). They are glycoproteins that can exist as monomers, dimers, or tetramers. These copper-containing enzymes facilitate biodegradation as they can catalyse the one-electron oxidation of a wide variety of aromatic and non-aromatic substrates, such as phenols, anilines, arylamines, thiols, monolignols, and lignins, while simultaneously reducing molecular oxygen to water in a four-electron process (**Berthet et al., 2012**). For many years, the potential role of laccases in lignin polymerization has been suggested by multiple studies (**Bao et al., 1993; Dean and Eriksson, 1994; Sterjiades et al., 1993**). However, to date, no research has been conducted on this enzyme family in the lignocellulosic fibre-producing crop plant-jute (*Corcorus* sp.).

Jute is a commercial crop known for its long, strong, environment friendly and multi utility lignocellulosic bast (phloem) fibres. As these fibres are coarse they are primarily used in the textile, paper, and packaging industries. The coarseness comes from the high lignin content (12-13% in

C. capsularis and 13-14% in *C. olitorius*) and limits their applications compared to other bast fibres like flax (*Linum usitatissimum*), which contains only about 2% lignin, and flower fibres like cotton (*Gossypium herbaceum*), which contain no lignin (**Majumder et al., 2020a**). Lignin content in jute fibres can be as high as 12-26% (**Selver et al., 2018**). Lowering lignin in jute fibres could increase their commercial market and versatile applications.

Research in plant molecular biology, aimed at reducing lignin content in jute fibres, requires a comprehensive understanding of the jute lignification process and the genes involved. The initial step in this challenging objective is the *in-silico* identification of relevant gene families and their members within the plant genome. This has become feasible with the availability of the jute reference genome. The genomes of both the commercially cultivated varieties of jute are now available: white jute (*C. capsularis*) has a genome size of 336 Mb and contains 25,874 genes, while tossa jute (*C. olitorius*) has a genome size of 361 Mb with 28,479 genes (**Zhang et al., 2021**). Recently, six members of the Caffeoyl-CoA 3-O-methyltransferase (*CCoAOMT*) gene family, which are involved in the jute lignin pathway, have been identified through *in-silico* studies (**Akhter et al., 2022)**. Laccase (*LAC*) and peroxidase (*PER*) are involved in the final steps of the lignification process, responsible for the polymerization of monolignols (**Boerjan et al., 2003**). It has been suggested that laccases play a potential role in assisting peroxidases during this final step. Specifically, laccases may function during the early stages of lignification to polymerize monolignols, while peroxidases may be more active in the later stages of stem development (**Sterjiades et al., 1993**). The role of peroxidases in the lignification process has been more extensively studied compared to laccases (**Berthet et al., 2012**). To date, no studies have been reported on the *LAC/PER* gene families in jute, although they have been reported in other plants.

In-silico analysis of a genome-wide search for laccase in *Arabidopsis thaliana* has identified 17 putative *LAC* genes (**McCaig et al., 2005**). In *Populus trichocarpa*, this number is 49 (**Lu et al., 2013**), while in *Eucalyptus grandis*, it is 54 (**Arcuri et al., 2020**). For *Linum usitatissimum*, 45 *LAC* genes have been identified (**Le Roy et al., 2017**), and in *G. hirsutum*, there are 84 *LAC* genes (**Balasubramanian et al., 2016**). These findings indicate that laccases in plants are encoded by medium to large multigene families. Functional validation of *LAC* gene family members involved in the lignification process has been reported in *Arabidopsis*. Among the 17 *AtLAC* (Arabidopsis thaliana Laccase) genes identified in *Arabidopsis*, *AtLAC4* and *AtLAC17* have

been shown to participate in the lignification process through gene knockdown experiments (**Berthet et al., 2011**). Additionally, overexpression of the cotton *GaLAC1* gene in poplar resulted in a 13-fold increase in laccase activity and a 2-19% rise in lignin content compared to non-transgenic controls (**Wang et al., 2008**). Such validations have helped identify specific gene family members involved in lignification, which can be further utilised to regulate lignin expression in plants.

The identification process often begins with tissue-specific expression analysis of gene family members. Since stems predominantly contain lignified tissues, it is hypothesised that stem-specific laccases could have redundant functions and be involved in the lignification process. In *Arabidopsis*, *AtLAC2, AtLAC4, AtLAC10, AtLAC11, AtLAC16,* and *AtLAC17* were found to be highly expressed in stem tissues compared to seed and root tissues, with *AtLAC4* and *AtLAC17* being directly involved in lignification (**Berthet et al., 2011; 2012**). To understand the lignification process in jute fibres, it is crucial to study the phloem (bast) tissues of the jute stem.

Mutant cultivars play a crucial role in the functional validation of genes. In the case of model plants like *A. thaliana*, *LAC* gene-specific knockout mutants are available for study. However, such resources are not available for crops like jute. Fortunately, an X-ray-irradiated mutant white jute line- *dlpf* (deficient lignified phloem fibre) with 50% lower lignin content compared to its control cultivar JRC212, was available (**Sengupta and Palit, 2004**). Transcriptomic analysis of the *dlpf* mutant and JRC212 control jute revealed lower expression of multiple genes in the phenylpropanoid pathway, including the lignin pathway (**Chakraborty et al., 2015**). A comparative expression study of the white jute *LAC* gene (*CcaLAC*) in *dlpf* and JRC212 throughout their developmental stages is sure to provide valuable insights into their involvement in the lignification process.

Our objective stands to identify all members of the *CcaLAC* gene family in white jute and analyse their expression in various tissues and developmental stages. This will help us pinpoint the *LAC* genes involved in the lignification process for further functional studies.

2. Materials and methods

2.1. White jute genome sequence

The white jute (*C. capsularis*) genome was downloaded from the Genome Warehouse (https://ngdc.cncb.ac.cn/search/specific?db=&q=GWHBCLC00000000, accessed on November 28, 2023) as per the information provided by **Zhang et al. (2021)**. Files containing DNA sequences, protein-coding sequences (CDS), and protein sequences were retrieved for *in-silico* analysis.

2.2. Plant material

Seeds of the popular white jute JRC321 *dlpf* mutant variety (known for its finest fibre quality with 50% lower lignin content) and its control JRC212 were collected from the Central Research Institute for Jute and Allied Fibres, Kolkata, West Bengal, India, for this study. The plants were grown under greenhouse conditions (30±2°C, 80% humidity, and a 13-hour light/11-hour dark cycle) for the necessary experiments. Four types of tissue samples (leaf, root, xylem, and phloem) were collected from JRC321 plants, 60 days after sowing (DAS) to study tissue-specific *LAC* gene expression. Additionally, phloem tissue was collected from JRC212 and *dlpf* plants at 30, 60, 90, and 120 DAS to examine *CcaLAC* gene expression at different developmental stages. For abscisic acid (ABA) stress hormone and copper heavy metal stress experiments, 60 DAS JRC321 plants were used. Only stem tissue was collected from these abiotic-stressed plants. All samples were stored at -80°C until RNA isolation.

2.3. Identification of laccase in white jute genome and sequence analysis

Laccase protein sequences from three plants—*A. thaliana, Oryza sativa, and Theobroma cacao*—were extracted from TAIR (**Lamesch et al., 2012**), Oryzabase (**Kurata and Yamazaki, 2006**), and the Phytozome database (**Goodstein et al., 2012**), respectively. A total of 74 *LAC* sequences from these three plants were used as references to identify *CcaLAC* genes in white jute by creating a Hidden Markov Model (HMM) profile. The HMM profile was generated using the HMMER tool

(version 3.2), and a search was conducted against the pre-downloaded jute protein dataset to identify putative *CcaLAC* genes (**Finn et al., 2011**).

The presence of known laccase protein family-specific domains (PF00394.19, PF07731.11, and PF07732.12) in the putative CcaLAC sequences was confirmed using either NCBI CDD (**Marchler-Bauer et al., 2015**), SMART (**Letunic et al., 2021**), or HMM Scan (**Finn et al., 2011**). Sequence hits with redundancy cut-off values greater than 0.01 were removed from the analysis, leaving all non-redundant hits (**Jha et al., 2024**). The physical and chemical properties of the CcaLAC proteins were analysed using the ProtParam tool on the Expasy server (**Wilkins et al., 1999**). The transmembrane nature of the CcaLAC proteins was assessed using the TMHMM-2.0 tool (**Krogh et al., 2001**).

2.4. Chromosomal mapping

A physical mapping of the *CcaLAC* genes, based on their genomic coordinates, was obtained using MapInspect software version 1.0 (https://mapinspect.software.informer.com, accessed on November 28, 2023).

2.5. Phylogeny analysis

For phylogenetic analysis, the CcaLAC protein sequences from *A. thaliana, O. sativa,* and *T. cacao* and white jute (*C. capsularis*) were used. The sequences were aligned using MUSCLE (**Edgar, 2004**), followed by the generation of phylogenetic trees with MEGA version 10.0 (**Kumar et al., 2018**). The maximum likelihood method was applied for the phylogenetic trees with 1000 bootstrap replications, gap deletions, and site-gamma (G) distribution rate as parameters (**Jha et al., 2024**).

2.6 Motif analysis

The conserved motifs in CcaLAC proteins were analysed using the MEME suite program (**Bailey et al., 2009**). The parameters for predicting conserved motifs were set with a maximum number of motifs at 10, a minimum width of 6, and a maximum width of 50 (**Chanwala et al., 2023**).

2.7. Gene structure and gene duplication analysis

The exon-intron structures of each *CcaLAC* gene were determined using the Gene Structure Display Server tool (**Hu et al., 2015**). Mutations such as gene duplication and base substitution rates were analysed for the identified *CcaLAC* genes. Duplication events were assessed with MCScanX (**Wang et al., 2012**), and synonymous (Ks) and nonsynonymous (Ka) substitution rates were determined using PAL2NAL (**Suyama et al., 2006**).

2.8. Synteny analysis

The synteny relationships between *CcaLAC* genes of white jute and *LAC* genes from other plants, such as *A. thaliana, Oryza sativa,* and *T. cacao,* were analysed and visualised using AccuSyn software (**Siri et al., 2020**).

2.9. Cis-element analysis

The upstream regions (≈2000 bp) of *CcaLAC* genes were extracted from the *C. capsularis* genome for cis-element analysis. Regulatory cis-elements were identified using the PlantCARE database (**Rombauts et al., 1999**). The analysis focused on identifying cis-elements associated with abiotic and biotic stress responses, cell cycle regulation, circadian rhythms, growth and development, physiology, metabolism, and hormonal signalling.

2.10. *In-silico* expression analysis of *LAC* genes in Jute

The CDS sequences of the 34 *CcaLAC* genes were used as a reference for mapping the short reads generated from the bast (phloem) tissue of control JRC212 and mutant *dlpf* genotypes of white jute. The raw reads for both these samples were obtained from NCBI Sequence Read Archive (SRA) database (Accession number SRR1585372 for JRC212 and Accession number SRR1585373 for mutant *dlpf*) and transcript quantification for the laccase CDS was carried out using the scripts align_and_estimate_abundance.pl and matrix was generated using the script abundance_estimates_to_matrix.pl included in the Trinity rnaseq package (https://github.com/trinityrnaseq/trinityrnaseq, accessed on July 10, 2024). The resulting TMM normalised read counts were converted to their log2 values and a heatmap was created with MeV software (https://sourceforge.net/projects/mev-tm4, accessed on July 10, 2024).

2.11. micro RAN (miRNA) target findings on *CcaLAC*

The mature micro-RNA sequences of *A. thaliana* were downloaded from PmiREN2.0 (**Guo et al., 2022**). The candidate *CcaLAC* sequences, along with the Arabidopsis miRNA sequences, were then uploaded to psRNATarget to predict the target sites on the identified laccase genes (**Dai and Zhao, 2011**).

2.12. Abiotic stress treatments

Abscisic acid (ABA) hormone stress (0.15 mM) treatments and copper heavy metal stress (0.10 mM $CuSO_4 \cdot H_2O$) were administered to 60-day-old, greenhouse-grown, healthy JRC321 plants following the methodology described by **Parida et al. (2024**). Samples were collected at six time points: 0 h, 2 h, 4 h, 8 h, 12 h, and 24 h, with stem tissues being used. Three individual plants were utilised as biological replicates for each time point.

2.13. RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues (leaf, xylem, phloem, and root) using a commercial RNA isolation kit (Macherey-Nagel, Germany). According to the manufacturer's protocol, 100 mg of tissue sample was ground into a powder using liquid nitrogen in a mortar and pestle. To eliminate any DNA contamination, a DNase treatment step was performed as instructed by the kit. The integrity of the RNA was assessed using 2% agarose gel electrophoresis, and RNA concentration was measured with a NanoDrop-2000c spectrophotometer (ThermoScientific, USA). First-strand cDNA was synthesised from 1 µg of RNA using a first-strand cDNA synthesis kit (ThermoScientific, USA) following the kit provided protocol.

2.14. PCR Primers

Primers for this study were designed using the PrimerQuest tool provided by Integrated DNA Technologies (IDT), USA (https://www.idtdna.com/PrimerQuest). Default parameters for qRT-PCR primer design were used, which include a melting temperature of 59–62 °C, GC content of 35–65%, primer length of 17–30 bp, and an amplicon size of 75–150 bp. The detailed list of primers is available in the **Supplementary Table 1**.

2.15. Gene expression analysis in qRT-PCR

The QuantStudio™ 5 system (ThermoScientific, USA) equipped with QuantStudio™ Design and Analysis Software version 1.5.2 was used to conduct the qRT-PCR analysis. Freshly synthesised cDNA was diluted 1:9 with molecular-grade water before being used as a sample in qRT-PCR. The experimental PCR cycle conditions were as follows: initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds, annealing at the primer-specific temperature (as listed in **Supplementary Table 1**) for 1 minute, and extension at 95 °C for 15 seconds. The melt curve of the amplified PCR products was obtained from 60 °C to 95 °C. The jute ubiquitin1 (*UBI*) gene (GenBank accession GH985256) was used as an internal control (**Parida et al., 2024**). The mRNA transcript expression levels were calculated and represented by relative quantification (RQ) values according to the method described by **Livak and Schmittgen (2001)**. Each qRT-PCR

analysis was performed in three technical replicates for each biological replicate, with three biological replicates used in total.

2.16. Homologous gene expression analysis

Eight *Arabidopsis* genes involved in the lignification process, namely *AtLAC2* (AT2G29130), *AtLAC4* (AT2G38080), *AtLAC5* (AT2G40370), *AtLAC10* (AT5G01190), *AtLAC11* (AT5G03260), *AtLAC12* (AT5G05390), *AtLAC15* (AT5G48100), and *AtLAC17* (AT5G60020) were selected based on previous research (**Berthet et al., 2012**). These *AtLAC* genes were used as queries in a BLAST search to identify their homologous genes in the jute genome using the TBtools software (**Chen et al., 2020**). *CcaLAC* genes that showed greater than 60% identity with the *AtLAC* sequences were considered homologous and selected for further qRT-PCR based expression analysis. The expression of these homologous *CcaLAC* genes was tested in the phloem and xylem tissues at different developmental stages (30 DAS, 60 DAS, 90 DAS, and 120 DAS) to understand the lignification process in white jute plants.

2.17. Statistical analysis

The mean Ct values were calculated using Microsoft Excel. For a comprehensive analysis, we performed a one-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism statistical software (version 9.0).

3. Results

3.1. Number of white jute laccase (*CcaLAC*s)

A total of 34 laccase proteins were identified in the genome of white jute (*C. capsularis*), named *CcaLAC1* to *CcaLAC34* based on their coordinates. The identified *LAC* genes were unevenly distributed across the seven chromosomes (**Figure 1**). The highest number, 10 *LAC* genes

(*CcaLAC25* to *CcaLAC34*), were located on chromosome 7, followed by chromosome 1 with 7 genes (*CcaLAC1* to *CcaLAC7*) and chromosome 2 with 6 genes (*CcaLAC8* to *CcaLAC13*). Three *LAC* genes were observed on chromosomes 4, 5, and 6 each, while two genes were present on chromosome 3.

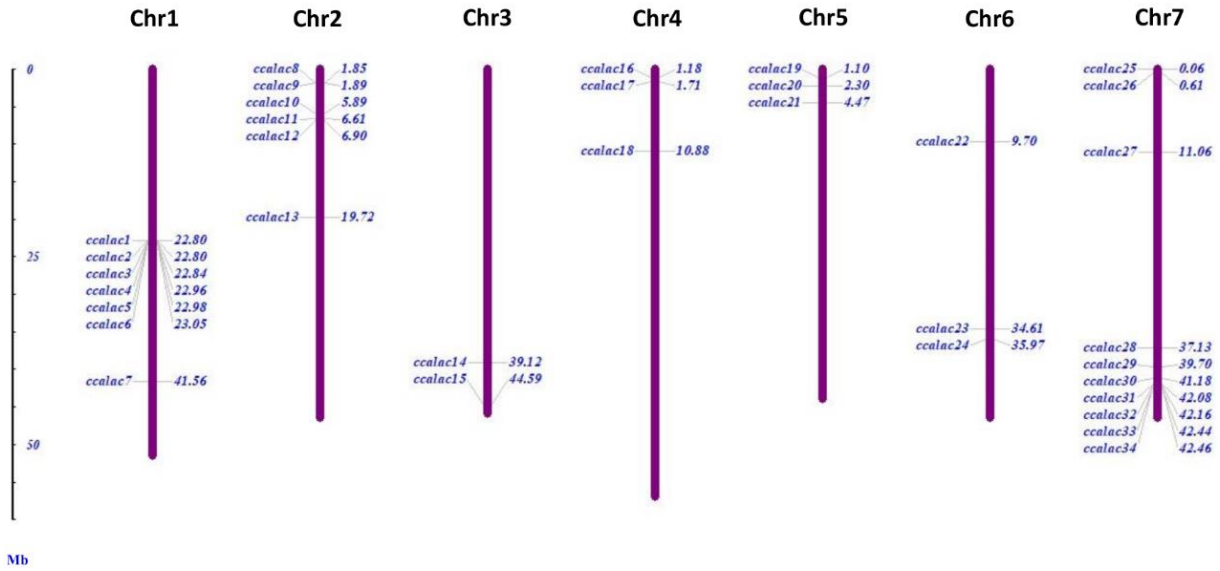

Figure 1. Chromosomal mapping of 34 laccase (*CcaLAC*) members in the white jute (*C. capsularis*) genome.

3.2. Laccase proteins

These CcaLAC proteins varied in length, ranging from 11.65 kDa (CcaLAC29) to 177.31 kDa (CcaLAC26). The highest number of amino acids in the CcaLAC proteins was 1583, the lowest was 102, and the average number of amino acids was approximately 610 (**Supplementary Table 2**). The instability index of the identified LAC proteins was predicted to be less than 40, except for CcaLAC19 (41.63) and CcaLAC26 (43.34), suggesting the stable nature of most of the proteins. Additionally, the aliphatic amino acid index of CcaLAC29 (65.1) was the lowest, while CcaLAC26 (93.14) was the highest. The grand average of hydropathy (GRAVY) value for all the CcaLACs was less than zero (<0), indicating their hydrophilic nature (**Kyte and Doolittle, 1982**). The identified CcaLAC proteins were predicted to be localised in various organelles of white jute (**Supplementary Table 2**). We found that 52.94% (18 out of 34) of CcaLACs were predicted to be transmembrane proteins (**Supplementary Figure 1**).

3.3. Motif of laccase proteins

The identified laccase proteins were predicted to comprise 10 motifs, designated as motif-1 to motif-10 (**Figure 2**). All the motifs, except for motif-3 and motif-6, were present in the majority of the LAC proteins. CcaLAC19 and CcaLAC29 contained only 3 and 1 motifs, respectively. Additionally, the CcaLAC2, CcaLAC3, and CcaLAC10 laccase proteins showed repetitions of certain motifs.

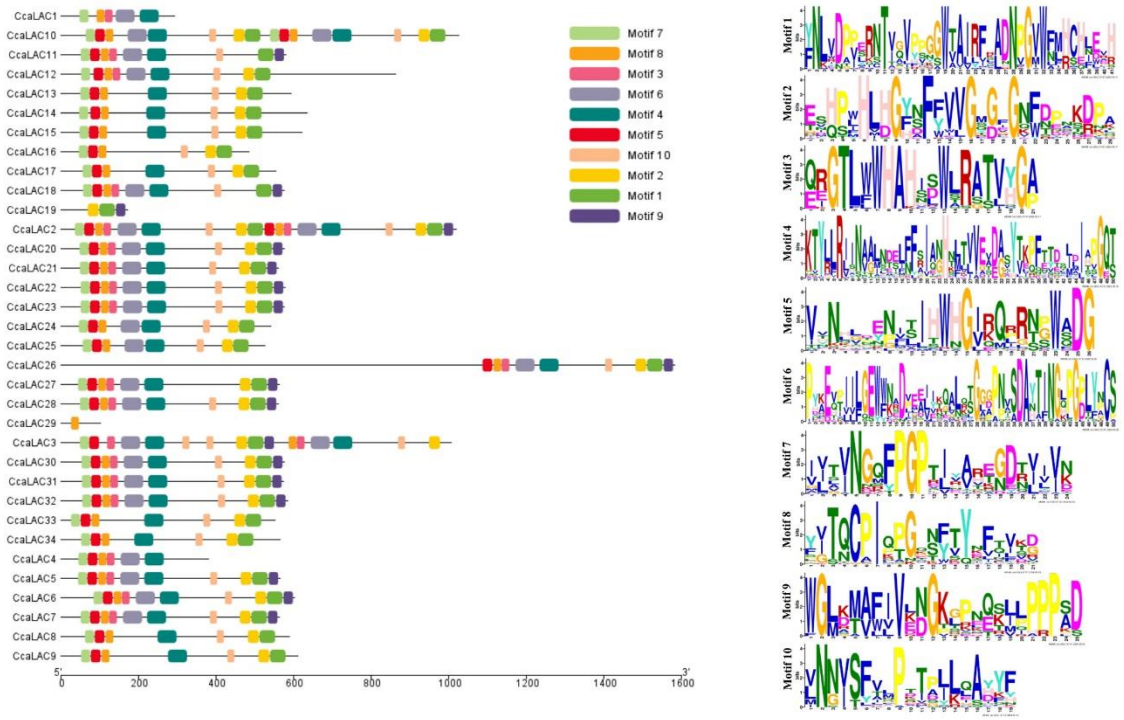

Figure 2. Conserved motif distribution in identified CcaLAC proteins. Motifs are indicated by different colours and their respective sequences.

3.4. *CcaLACs* gene structure

The identified *CcaLAC* genes had large variations in exon-intron distribution, ranging from 2-28 numbers of exons. Two exons were present in the *CcaLAC19*, whereas the highest number (28) of exons were predicted in the *CcaLAC26* gene (**Figure 3**).

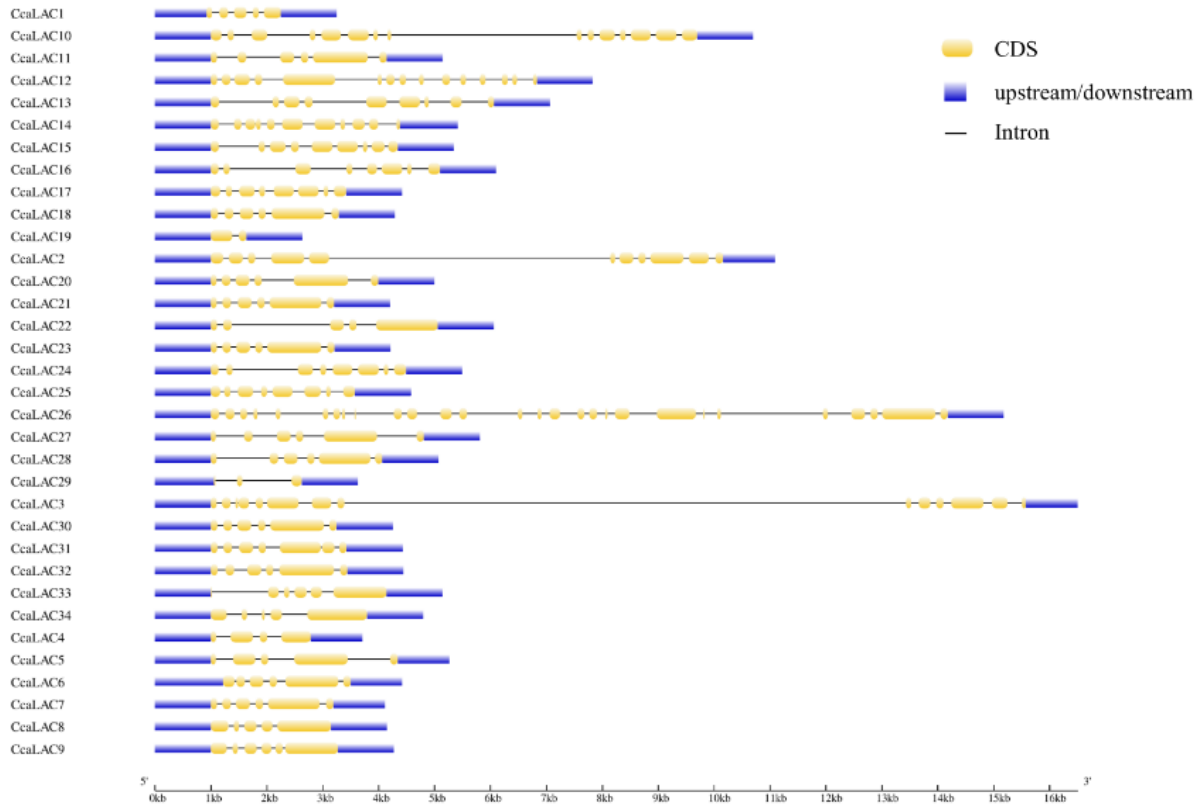

Figure 3. Gene structure of 34 white jute *laccase* genes (CcaLACs).

3.5. Phylogeny of CcaLACs proteins

The phylogeny distributed the identified laccase proteins of white jute into six groups, named Group I to Group VI (**Figure 4**). The majority of laccase proteins were found in Group IV (16 laccase proteins), Group I (9 laccase proteins), and Group III (7 laccase proteins). Group II and Group V had one member each.

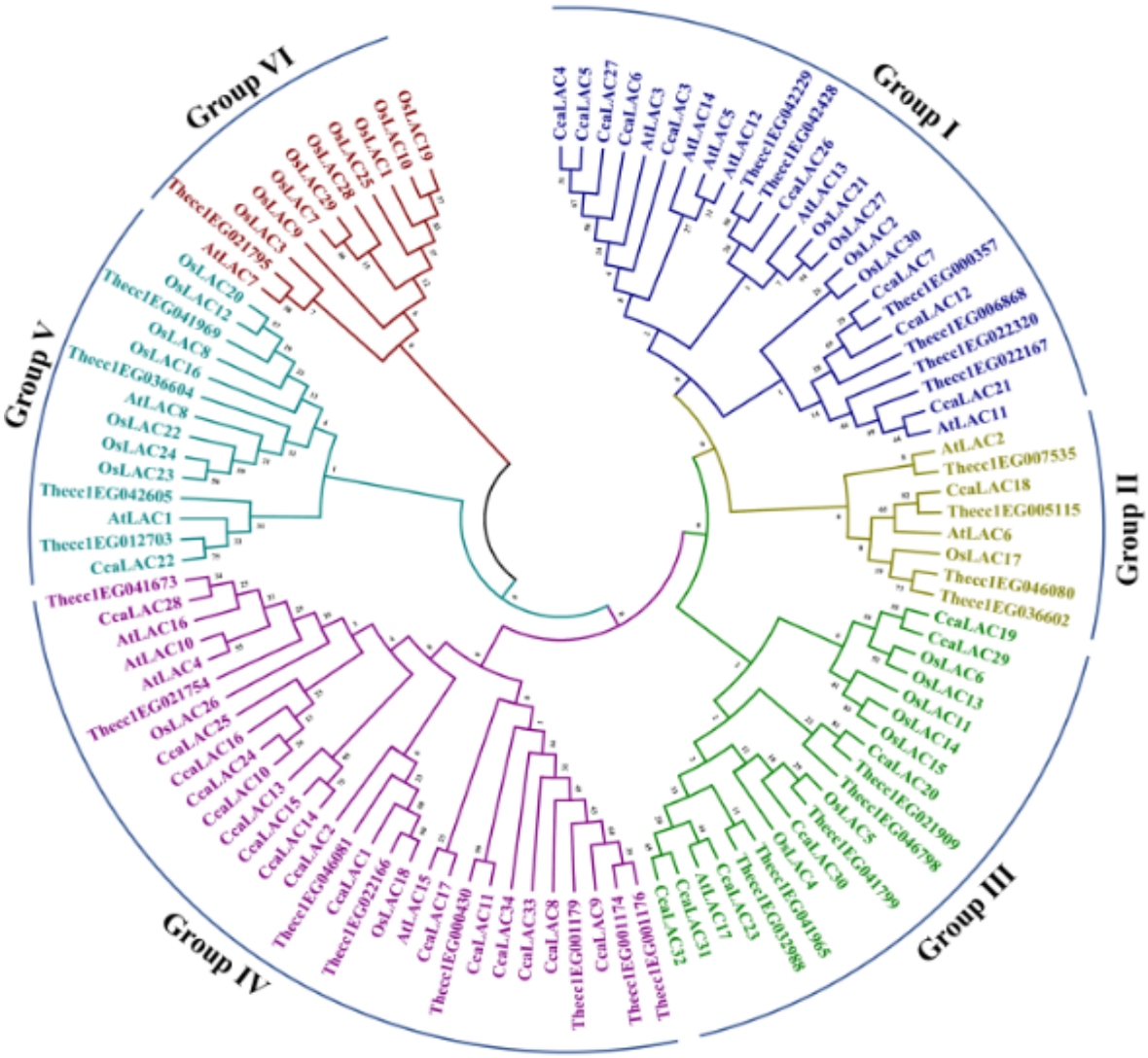

Figure 4. Phylogenetic tree of jute LAC proteins with LAC proteins of *A. thaliana*, *O. sativa*, and *T. cacao*. The phylogeny classified the identified LAC members into six groups.

3.6. *CcaLAC* genes duplication

We identified 20 paralogous pairs from gene duplication analysis, comprising 4 tandem pairs and 16 segmental pairs (**Supplementary Table 3**). Additionally, 96 orthologous pairs were identified, which included 21 pairs between *C. capsularis* and *A. thaliana*, 21 pairs between *C. capsularis* and *O. sativa*, and 54 pairs between *C. capsularis* and *T. cacao* (**Figure 5**). Maximum number of

pairs of *LAC* was found between jute and cacao, likely due to their high genome similarity, as reported by **Sarkar et al. (2017)** and **Zhang et al. (2021)**.

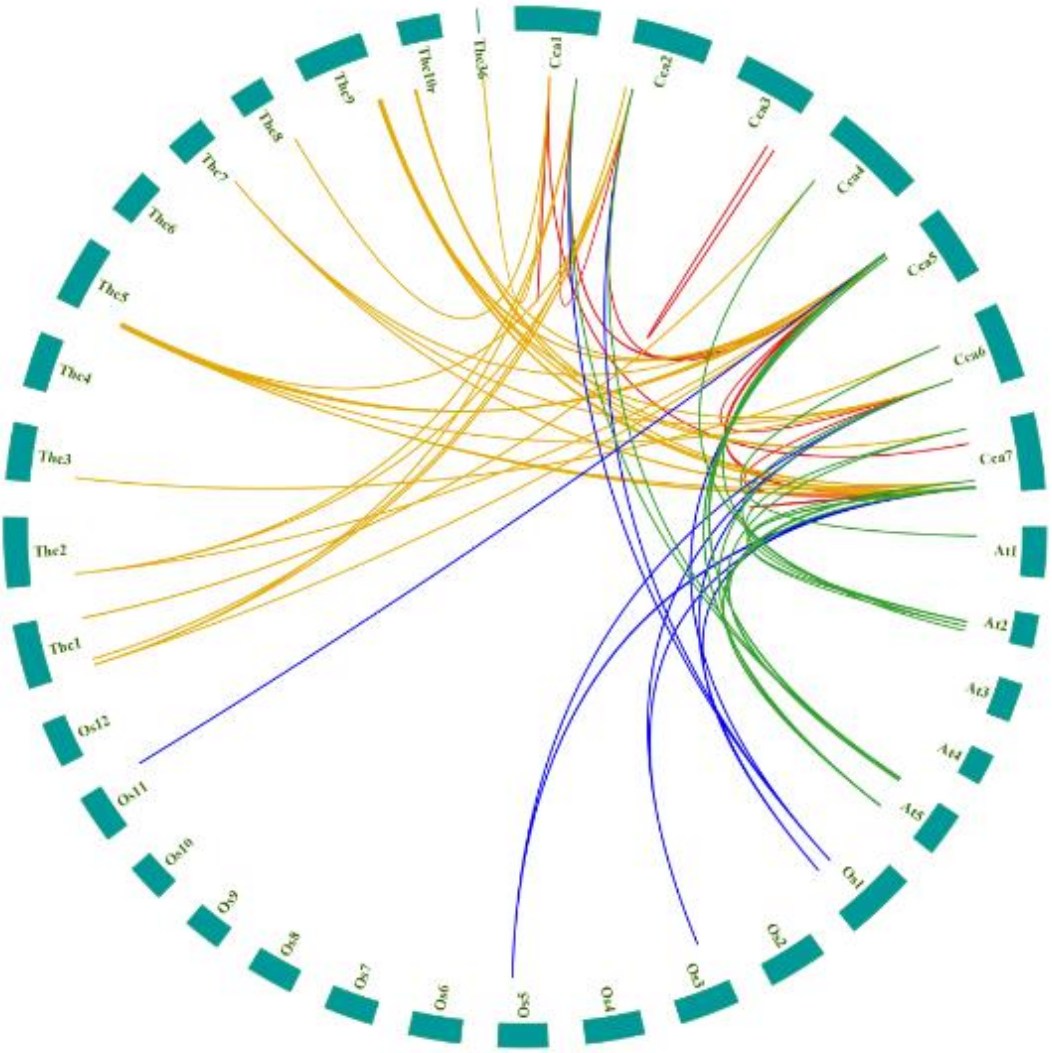

Figure 5. Synteny relationship between white jute (*C. capsularis*), Arabidopsis (*A. thaliana*), rice (*O. sativa*), and cacao beans (*T. cacao*) *LAC* genes. Each coloured line represents duplicated gene pairs among the species.

3.7. Cis-acting elements

Many diverse cis-elements were predicted in the upstream region of *CcaLAC* genes (**Supplementary Figure 2**). Cis-elements like MYB, MYC, and TATA-box were present in all 34 identified members. Cis-elements associated with various biological functions, such as stress (STRE, W-box, MYB, MYC, WUN-motif), hormonal signalling (ABRE, ERE, GARE-motif, P-box, TGA element), tissue specificity, and developmental processes (G-box, GT1, RY-element, Sp1, GATA-motif, LAMP element, Box-4, TCT-motif), were predominantly found in the upstream region of *CcaLAC* genes, suggesting their diverse biological functions (**ain-Ali et al., 2021; Jha et al., 2024**).

3.8. Tissue specific expression of *CcaLAC* genes

Normalised expression of all the 34 *CcaLAC* genes in four different tissues (leaf, phloem, xylem and root) was examined using qRT-PCR. The mRNA transcript expression levels are represented by relative quantification (RQ) values, with root tissue expression set as the reference (RQ value 1) (**Figure 6**). The following observations were made: Higher expression in leaf tissue compared to other tissues was observed for *CcaLAC3, CcaLAC12, CcaLAC17, CcaLAC22, CcaLAC23, CcaLAC27, CcaLAC29, CcaLAC32,* and *CcaLAC33*. Elevated expression in phloem tissue was noted for *CcaLAC2, CcaLAC6, CcaLAC7, CcaLAC8, CcaLAC9, CcaLAC11, CcaLAC15, CcaLAC19, CcaLAC20, CcaLAC21, CcaLAC24, CcaLAC25, CcaLAC26, CcaLAC28, CcaLAC30, CcaLAC31* and *CcaLAC34.* Higher expression in xylem tissue was recorded for *CcaLAC4, CcaLAC5, CcaLAC16,* and *CcaLAC18.* Increased expression in root tissue was found for *CcaLAC1, CcaLAC10, CcaLAC13,* and *CcaLAC14.*

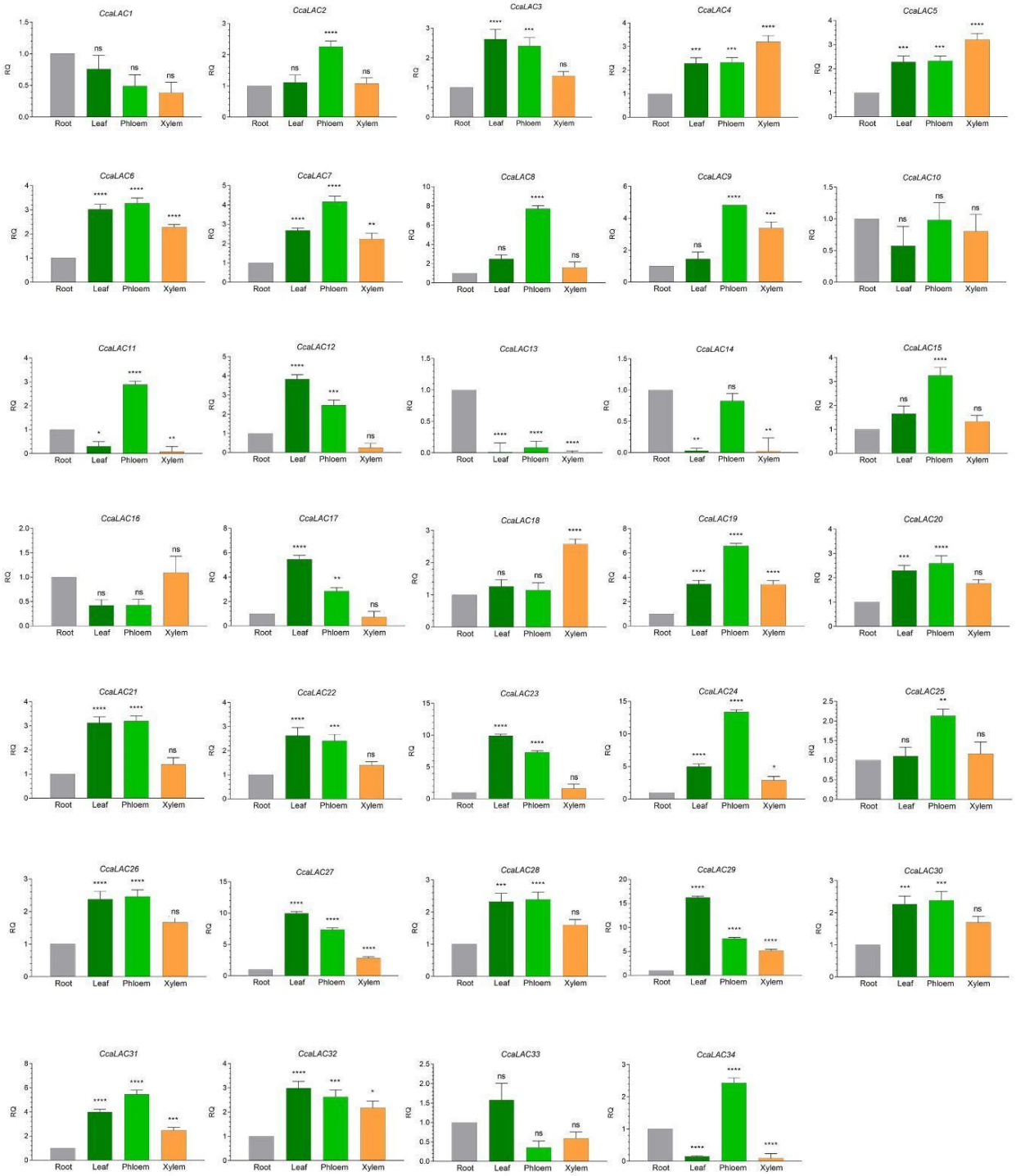

Figure 6. Tissue-specific expression of laccase (*CcaLAC*) genes. This figure shows the relative quantification (RQ) values of *CcaLAC* gene expression in the leaf, phloem, xylem, and root tissues of white jute. The RQ values are normalised using the ubiquitin 1 (*UBI*) housekeeping gene. A

one-way ANOVA (*F*-test) with a significance threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was performed. Asterisks denote significant differences in *P* values from Tukey's test, while non-significant values are marked as 'ns'. The data represent three biological replicates.

3.9. *AtLAC* homologous *CcaLAC* gene expression at different developmental stages

Jute is primarily cultivated for its phloem (bast) fibres. Analysing the expression of phloem-specific *AtLAC* homologous *CcaLAC* genes at four different time points—30 DAS (when fibre development initiates), 60 DAS, 90 DAS, and 120 DAS (when the plant is harvested for fibre isolation)—provides insight into the lignification process. *CcaLAC19, CcaLAC21, CcaLAC23, CcaLAC28, CcaLAC29,* and *CcaLAC32* showed a steady increase in expression from 30 DAS to 120 DAS (**Figure 7**). *CcaLAC12, CcaLAC20, CcaLAC26* and *CcaLAC30* showed a steady increase in expression up to 90 DAS, followed by a sharp drop at 120 DAS. In contrast, *CcaLAC7* and *CcaLAC31* exhibited a slight decrease in expression at 120 DAS. For all the tested *AtLAC* homologous genes in the lignin pathway, expression in phloem tissue increased from 30 DAS to 90 DAS.

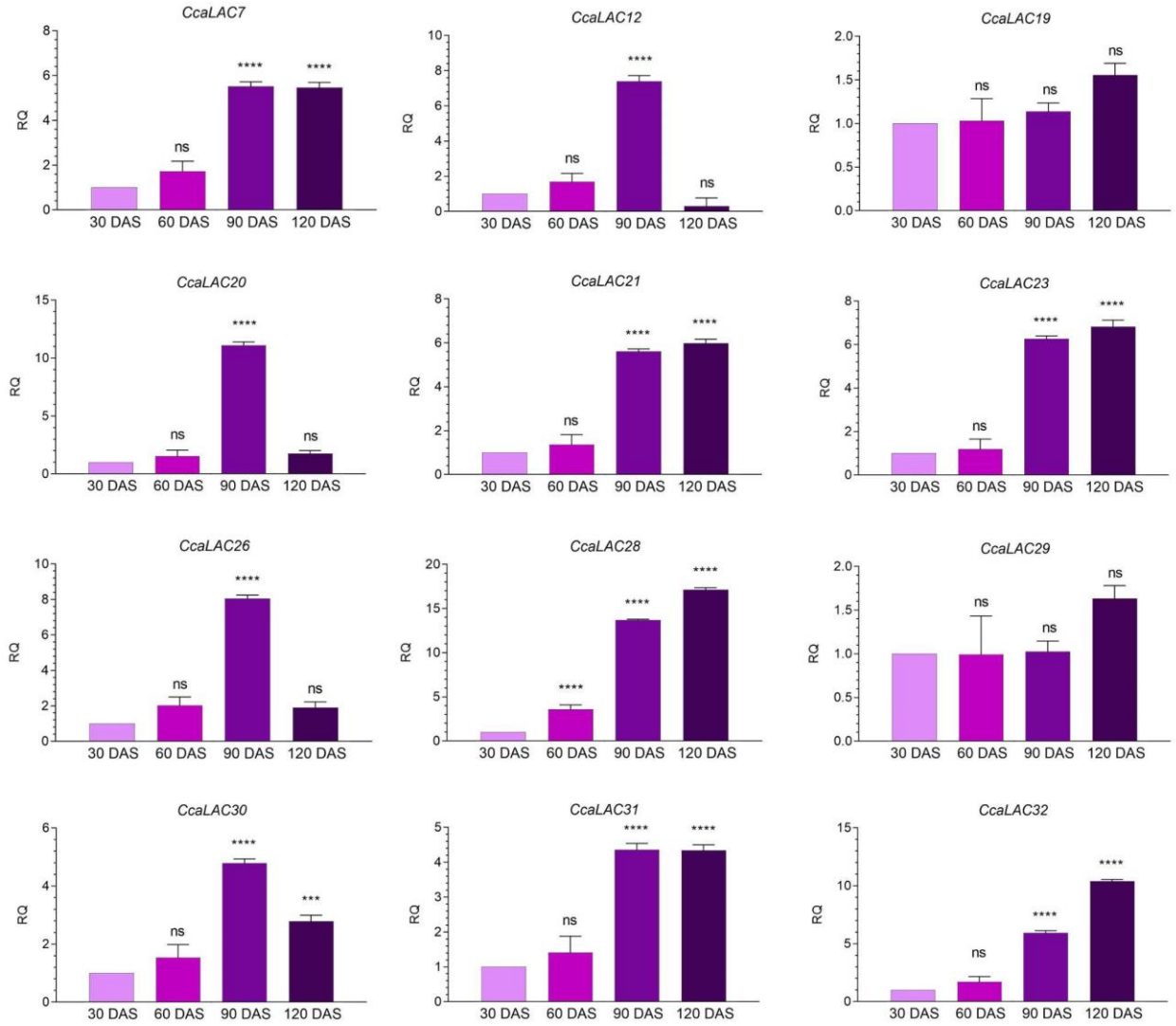

Figure 7. Expression of laccase (*CcaLAC*) genes during developmental stages of white jute plants. This figure illustrates the expression of *CcaLAC*s in the phloem tissue of white jute at four different time points, from 30 to 120 days after sowing (DAS). A one-way ANOVA (*F*-test) with a significance threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was performed. Asterisks denote significant differences in *P* values from Tukey's test, while non-significant values are marked as 'ns'. The data represent three biological replicates.

3.10. *In-silico* expression analysis of CcaLACs

In-silico expression analysis of *CcaLAC* genes was performed using available transcriptomics data from 30 DAS phloem tissue of JRC212 and its X-ray-irradiated mutant line *dlpf*. RNAseq raw reads of phloem tissue for JRC212 and *dlpf* were sourced from the study by **Chakraborty et al. (2015)**. The expression of all 34 laccase genes was observed in phloem tissue (**Figure 8**). In the *dlpf* mutant, the expression of *CcaLAC3, CcaLAC4, CcaLAC7, CcaLAC9, CcaLAC17, CcaLAC18, CcaLAC21, CcaLAC23, CcaLAC31, CcaLAC32,* and *CcaLAC33* was found to be lower than in the control JRC212. Nine *CcaLAC* genes from the heatmap were randomly selected for revalidation of their expression via qRT-PCR. The qRT-PCR results demonstrated a similar expression pattern to that shown in the transcriptomics data heatmap (**Supplementary Figure 3**).

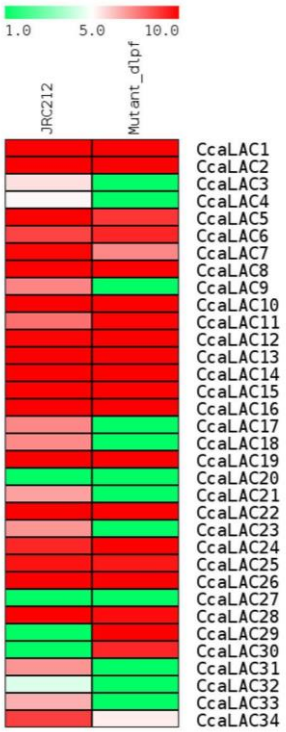

Figure 8. *In-silico* expression analysis of laccase genes (CcaLACs) in the phloem tissue of JRC212 (control) and dlpf (mutant) at 30 DAS. Heat map based on publicly available transcriptomics data from BioProject PRJNA255934. The expression scale ranges from low (1, green) to high (10, red).

3.11. *AtLAC* homologous *CcaLAC* gene expression in low lignin mutant jute (*dlpf*)

The expression patterns of *CcaLAC21*, *CcaLAC23*, *CcaLAC28*, and *CcaLAC32*, which showed a gradual increase in expression with plant age (30 to 120 DAS) in the control variety JRC212, were also examined in the low lignin *dlpf* mutant at the same developmental stages. A significant (P<0.0001) decrease in expression was observed in *dlpf* compared to JRC212 at 90 DAS and 120 DAS for these four genes (**Figure 9**). A similar decrease in expression was noted in *dlpf* at 90 DAS for *CcaLAC12*, *CcaLAC20*, and *CcaLAC26* genes. These three genes showed high expression in JRC212 at 90 DAS compared to other time points but did not show such expression in *dlpf* (**Supplementary Figure 4**). Two genes, *CcaLAC19* and *CcaLAC29*, exhibited minimal expression in JRC212 at all time points and nonsignificant differences in expression in *dlpf* compared to JRC212 (**Supplementary Figure 4**). This comparison aims to elucidate the differences in *CcaLAC* gene expression patterns between the JRC212 control and the *dlpf* mutant jute.

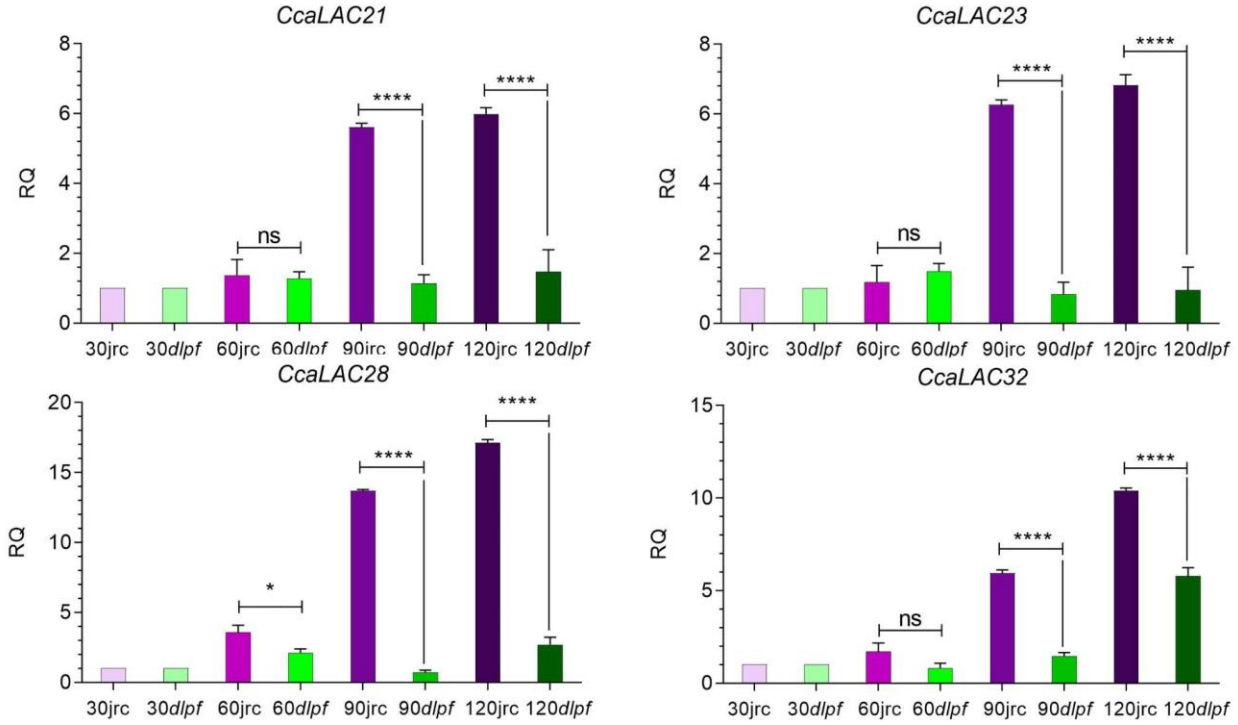

Figure 9. Comparative study of laccase (*CcaLAC*) gene expression in white jute cultivar JRC212 and its mutant line *dlpf*. This figure presents the comparative expression analysis of laccase (*CcaLAC*) genes between JRC212 and *dlpf* (which has fibres with 50% lower lignin content), across different developmental stages (30 DAS to 120 DAS). A one-way ANOVA (F-test) with a significance threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was performed.

Asterisks denote significant differences in *P* values from Tukey's test, while non-significant values are marked as 'ns'. The data represent three biological replicates.

3.12. Expression of *CcaLAC* under copper stress

The expression of two highly expressive genes, *CcaLAC28* and *CcaLAC32*, homologous to *AtLAC4* and *AtLAC17* respectively, which are involved in the lignin synthesis pathway in Arabidopsis, was examined in plants subjected to copper heavy metal stress. In *CcaLAC28*, a significant increase in expression was observed at 12 hours, whereas in *CcaLAC32*, all time points from 2 hours to 24 hours showed significantly high expression under copper heavy metal stress (**Figure 10A**). For *CcaLAC32*, the expression gradually increased up to 8 hours and then decreased.

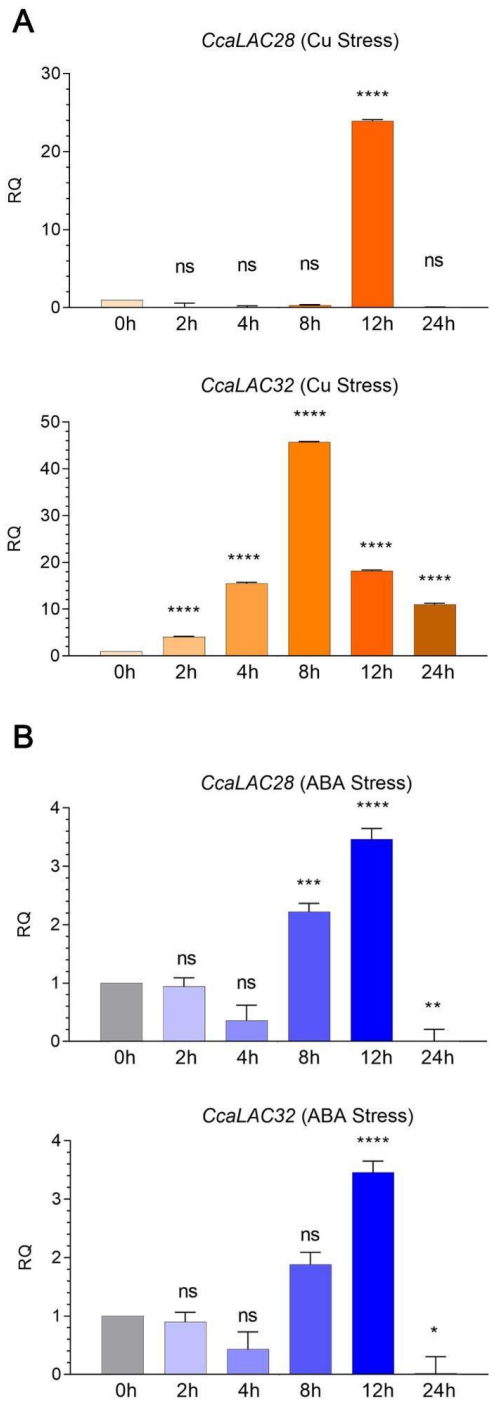

Figure 10. Expression of *CcaLAC28* and *CcaLAC32* under abiotic stresses. This figure depicts the expression levels of *CcaLAC28* and *CcaLAC32* genes in white jute plants subjected to (A) copper stress using (0.10 mM $CuSO_4 \cdot H_2O$), (B) ABA hormone stress. A one-way ANOVA (F-test) with a significance threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was

performed. Asterisks denote significant differences in *P* values from Tukey's test, while non-significant values are marked as 'ns'. The data represent three biological replicates.

3.13. Expression of *CcaLAC* under ABA hormonal stress

In plants treated with ABA hormone stress, the expression of the *AtLAC* homologous *CcaLAC* genes, *CcaLAC28* and *CcaLAC32*, was recorded. A significant increase in gene expression was observed at 8 h and 12 h for both genes (**Figure 10B**). The highest expression was observed at 12 h, while the lowest expression was recorded at 24 h. The gene expression patterns of *CcaLAC28* and *CcaLAC32* suggest that they act as delayed responsive genes in white jute under ABA hormonal stress.

3.14. *CcaLAC* targeted by micro RAN (miRNA)

White jute laccase genes (*CcaLACs*) were predicted to be target sites for commonly known lignin-related miRNAs (**Supplementary Table 4**), specifically miR397. Ath-miR397a was found to target *CcaLAC2, CcaLAC3, CcaLAC7, CcaLAC20, CcaLAC21, CcaLAC22, CcaLAC23, CcaLAC26, CcaLAC28, CcaLAC30,* and *CcaLAC31*, whereas Ath-miR397b was predicted to target *CcaLAC7, CcaLAC20, CcaLAC21, CcaLAC22, CcaLAC23, CcaLAC28, CcaLAC30, CcaLAC31*, and *CcaLAC34* (**Supplementary Figure 5**).

4. Discussion

The white jute genome contains 25,874 genes (**Zhang et al., 2021**), including the laccase (*CcaLAC*) genes, which remains unexplored especially regarding its function in stem development, fibre lignification, and performance under abiotic and biotic stress. This study aims to explore the role of *LAC* in white jute (*C. capsularis*). We identified 34 *CcaLAC* genes in the white jute genome and examined their expression in different tissues (**Figure 6**). Out of the 34 *CcaLAC* genes, 17 were highly expressed in phloem, 9 in leaves, 4 in xylem, and 4 in roots. Considering the stem part

as a combination of phloem and xylem tissue-specific expression, 61.76% of the *CcaLAC* genes are expressed here, which is highly significant in terms of the role of laccase in jute stem and fibre development. Such observations have been reported in *Arabidopsis*, where *AtLAC* genes (*AtLAC2, AtLAC4, AtLAC10, AtLAC11, AtLAC16,* and *AtLAC17*) responsible for the lignification process were found to be highly expressed in stem tissue (**Berthet et al., 2012**).

Separation of xylem and phloem tissue from the jute stem is very difficult in the initial days of its growth, but by 30 DAS, it becomes much easier, suggesting that fibre development and the lignification process have begun around this time (**Chakraborty et al., 2015**). By 110-120 DAS, phloem fibre can be easily extracted through the biological retting process, indicating its maturity and the completion of the lignification process (**Majumder et al., 2018a**). In this study, we targeted the time points of 30 DAS, 60 DAS, 90 DAS, and finally 120 DAS to relate *CcaLAC* expression to jute stem development and the fibre lignification process.

To investigate the potential involvement of *CcaLAC* in the fibre lignification process of jute, homologous genes from *Arabidopsis* were selected. *Arabidopsis* is commonly used for lignin pathway analysis in plants due to its lignified stem tissue, short life span, and its classification as a dicotyledonous plant (**Vanholme et al., 2010**). The *CcaLAC* genes showed highly similar sequences to *AtLAC* and their expression patterns also matched those of *Arabidopsis*, increasing with plant development (**Berthet et al., 2011**) (**Figure 7**).

Mutation of *AtLAC4* (homologous to *CcaLAC28*) and *AtLAC17* (homologous to *CcaLAC32*) individually in *Arabidopsis* showed a 10% reduction in lignin content, and when both genes (*AtLAC4+AtLAC17*) were mutated, lignin content was reduced by up to 39% (**Berthet et al., 2011**). This same study on the double mutant line also demonstrated that *AtLAC4* and *AtLAC17* contributed significantly to total laccase enzyme activity in the plants, with about a 50% reduction in laccase activity observed upon their mutation. Recently, overexpression of *AtLAC4* in populus increased lignin content in the transgenic populus without any phenotypic changes in the plants (**Ahlawat et al., 2024**). These findings established the involvement of *AtLAC* in the lignification process, specifically *AtLAC4* and *AtLAC17*. This suggests that their homologous genes, *CcaLAC28* and *CcaLAC32*, might also be involved in the lignification process of white jute.

The transcriptional expression of *CcaLAC* genes was tested at different developmental stages (30 DAS to 120 DAS) of jute plants through qRT-PCR estimation of mRNA transcripts. Four genes—*CcaLAC21, CcaLAC23, CcaLAC28,* and *CcaLAC32*—showed a steady increase in expression as the plant developed (**Figure 7**). This raised the question: Are these genes involved in the jute lignification process? To explore this, we examined the expression of these four *CcaLAC* genes at the same developmental stages in a low lignin fibre content mutant variety of white jute, *dlpf*. Upon comparing *dlpf* with its control, JRC212, we found statistically significant differences in the expression of these four *CcaLAC* genes. The lower expression of *CcaLAC21, CcaLAC23, CcaLAC28* and *CcaLAC32* could be a contributing factor to the low lignin concentration observed in *dlpf* (**Figure 9**).

The expression of *CcaLAC12, CcaLAC20,* and *CcaLAC26* was significantly higher at 90 DAS, but decreased at 120 DAS to levels similar to those observed at 30 DAS (**Supplementary Figure 4**). If these genes were solely involved in the lignification process, their expression should remain high until 120 DAS (like *CcaLAC21, CcaLAC23, CcaLAC28* and *CcaLAC32*). The observed decrease in expression suggests their potential role in stem elongation or structure, which might be achieved by 90 DAS. Further functional characterization is necessary to confirm this hypothesis. This expression pattern was also observed in the *dlpf* mutant (**Supplementary Figure 4**).

Conversely, *CcaLAC19* and *CcaLAC29* maintained baseline expression levels throughout the developmental stages in both the control JRC212 and its mutant, *dlpf*, with no significant changes (**Supplementary Figure 4**). This consistent expression suggests a fundamental role for these genes in white jute development.

Previous studies (up to 60 DAS) on the low-lignin mutant jute, *dlpf*, by **Chakraborty et al. (2015)** found a low expression of cinnamyl alcohol dehydrogenase (CAD), a key gene in the lignin pathway. Recently, we analysed the lignin pathway in *dlpf* (up to 120 DAS) comparing qRT-PCR expression data using two housekeeping genes-*UBI* and *beta-tubulin* (*BTUB*) (**Parida et al., 2024)**. We found lower expression of *CCoAoMT*, cinnamate 4-hydroxylase (*C4H*), and *CAD* in *dlpf* compared to its control JRC212. This study is the first to analyse the expression of *LAC* genes in low lignin mutant *dlpf*. The lower expression of *CcaLAC21, CcaLAC23, CcaLAC28* and

CcaLAC32 in *dlpf* may also contribute to the reduced lignin content, but further functional validation of these *CcaLAC* genes is needed (**Figure 9**).

The expression of plant laccases is controlled by transcriptional and post-transcriptional regulation. Transcriptional regulation of *LAC* through MYB transcription factors has been reported in multiple plants, which have one of the largest and most functionally diverse transcription factor families. In Arabidopsis, *AtMYB58* directly regulates *AtLAC4* expression; *AtMYB46* regulates *AtLAC10* and *AtHY5* regulates *AtLAC12* (**Zhou et al., 2009**). In *Phyllostachys edulis*, *PeMYB4.1/20/85.2* regulates *PeLAC20*; in *Pyrus bretschneideri*, *PbMYB26* regulates *PbLAC4*-like; and in *Pennisetum glaucum*, *PgMYB305* regulates *PgLAC14* (**Bai et al., 2023**). Analysis of cis-elements in the upstream sequences of jute laccases (*CcaLAC* genes) revealed a predominance of MYB binding sites in many of them (**Supplementary Figure 2**). MYB were found upstream of *CcaLAC19, CcaLAC21, CcaLAC23, CcaLAC28, CcaLAC29,* and *CcaLAC32,* indicating that these genes could be regulated by MYBs. Recently, 88 MYBs were identified in white jute through a whole genome search, but it remains to be determined which MYBs regulate *CcaLAC* expression (**Niyitanga et al., 2023**).

Post-transcriptional regulation of LAC by microRNAs (miRNAs) has been reported in multiple plants, including rice (*Oryza sativa*), Arabidopsis, and Populus. MicroRNA397 (miR397) is conserved in dicots, monocots, and gymnosperms, directly targeting laccase transcripts and controlling their expression (**Kozomara and Griffiths-Jones, 2011**). In Populus, 28 *PtLAC* genes are targeted by miR397 (**Lu et al., 2008**). In chickpea (*Cicer arietinum*) the *CaLAC4* and *CaLAC17L* genes are targeted by miR397 out of the 20 *CaLAC* genes (**Sharma et al., 2023**). The miR397 also regulates the expression of *OsLACs* in rice (**Bakhshi et al., 2016**). miR397 is considered a negative regulator of lignin biosynthesis by regulating *LAC* expression, as reported in rice, chickpea, Arabidopsis, and Populus (**Lu et al., 2013**).

Are the *CcaLACs* targeted by miR397? We found that miR397a targets 11 *CcaLAC*s, and miR397b targets 9 *CcaLAC*s in white jute (**Supplementary Figure 5**). *CcaLAC28* (homologous to *AtLAC4*) is targeted by both miR397a and miR397b, but no target site was found on *CcaLAC32* (homologous to *AtLAC17*). Overexpression of miR397 in jute, using the established methods of *Agrobacterium*-mediated transformation described by **Majumder et al. (2024a)**, could provide

more information on its involvement in the lignification process by regulating *CcaLACs* in the near future.

The presence of different cis-acting elements upstream (promoter region) of *CcaLAC*s indicates that their expression could be influenced by plant growth, developmental stages, hormones, and both abiotic and biotic stresses (**Supplementary Figure 2**). Cis-acting elements, specific to *CcaLAC28* (homologous to *AtLAC4*) and *CcaLAC32* (homologous to *AtLAC17*), are listed in **Supplementary Table 5**. The expression of these two genes was validated under two different stress conditions: ABA hormone and copper heavy metal stress. Although it is well-documented that laccase (*LAC*) genes are susceptible to environmental changes, with expression being up- or down-regulated by various abiotic factors (heavy metal ions, drought, salinity, temperature, oxidative stress) and biotic factors (pathogens, insects, fungi) (reviewed by **Bai et al., 2023**), no studies have yet examined the expression patterns of jute LAC under stress conditions.

Why were these (copper and ABA) specific abiotic stresses chosen? Copper is an essential heavy metal and a cofactor for laccase, playing a critical role in its catalytic function. Under copper (0.10 mM $CuSO_4 \cdot H_2O$) stress, significant differences in *LAC* gene expression were observed, with *CcaLAC28* showing a 23.96-fold increase at 12 h and *CcaLAC32* showing a 45.73-fold increase at 8 h compared to the 0 h samples (**Figure 10A**). Such high expression was also previously recorded in rice laccase 10 (*OsLAC10*) under copper stress, which showed a 1200-fold increase at 12 h (**Liu et al., 2017**).

ABA hormone stress mimics conditions of multiple stresses like salt, desiccation, and freezing in plants (**Sakata et al., 2014**). Additionally, literature supports that drought stress can alter the expression of laccase in plants (**Sharma et al., 2023; Turlapati et al., 2011**). In this study, we found that *CcaLAC28* (homologous to *AtLAC4*) and *CcaLAC32* (homologous to *AtLAC17*) responded to ABA stress (**Figure 10B**). The presence of cis-acting elements such as MYC, MYB, W-box, and CAT-box, which are known for ABA responsiveness in plants, likely explains this expression pattern (**Fujita et al., 2011**). These elements are also involved in drought and salinity stresses, suggesting that these genes might respond to those conditions as well. Further

studies on *CcaLAC* gene expression under various biotic and abiotic stresses will provide more information on LAC's upstream cis-acting elements and their interactions with stress.

The expression of *CcaLAC*s in plant cells (subcellular localization) was predicted *in-silico* (**Supplementary Table 2**). Nucleus-specific localization was predicted for five *CcaLACs* (*CcaLAC19, CcaLAC26, CcaLAC29, CcaLAC31,* and *CcaLAC32*), whereas nucleus and cytoplasm-specific localization were predicted for *CcaLAC12* and *CcaLAC34*. Subcellular localization of *CcaLACs* was also predicted for other organelles, including the plasma membrane, chloroplast, endoplasmic reticulum, Golgi apparatus, mitochondria, peroxisome, vacuoles, cytoskeletons, and extracellular spaces. The distribution of *CcaLACs* in cells was observed from the *in-silico* subcellular localization data. The presence of *CcaLACs* in all these membrane-bound compartments could be attributed to the presence of transmembrane helices in the majority (18 of 34) of *CcaLAC*s, as found in our analysis (**Supplementary Figure 1**). The structure of the plant laccase is still not well studied. Information about the presence of transmembrane helices could provide valuable insights into its structure.

Expression (up- and down-regulation) of *LAC* genes has been reported in multiple crops. In cotton, down-regulation of *GhLAC1* increases fibre initials and shortens fibre length (**Hu et al., 2020**). In common wheat (*Triticum aestivum*), silencing of *TaLAC4* resulted in a reduction in total lignin deposition in the stem (**Soni et al., 2020**). In pear (*Pyrus bretschneideri*), overexpression of *PbLAC1* boosted lignin production and cell wall growth (**Cheng et al., 2019**). In silvergrass (*Miscanthus* sp.), overexpression of *MsLAC1* developed higher lignin content (**He et al., 2019**). Downregulation of *CcaLACs* in white jute could alter its lignin content with or without any physiological changes. To avoid major physiological changes in jute plants due to the alteration of lignin content through downregulation of *CcaLAC*s, phloem-specific silencing of *CcaLAC*s can be possible in jute, using the AtSUC2 phloem specific promoter as described by **Majumder et al. (2020b)**. Transgenic research on jute is gaining momentum. Multiple agronomically important traits like insect resistance (IR) (**Majumder et al., 2018a, 2020c**), fungus resistance (FR) (**Majumder et al., 2018b**), herbicide tolerance (HT) (**Majumder et al., 2018b**), or a combination of these traits (IR-FR-HT) (**Majumder et al., 2024b**) have been developed for white jute (cultivar JRC321). Downregulation of lignin pathway genes (*COMT, C4H, C3H, F5H*) through RNAi has also been reported in tossa jute (**Nath et al., 2021**). Further study on the overexpression or

downregulation of selected *CcaLAC*s could develop low-lignin transgenic jute lines, fulfilling the long-standing industrial demand for low-lignin jute fibres. For such purposes, *CcaLAC28* and *CcaLAC32* could be potential candidate targets, but they require detailed functional validation.

5. Conclusion

The white jute (*C. capsularis*) genome contains 34 putative laccase genes (*CcaLAC1* to *CcLAC34*) distributed across its 7 chromosomes. The presence of these *CcaLAC* genes was validated using publicly available transcriptomics data of white jute. Most of these genes are expressed in phloem tissue, suggesting their likely involvement in fibre lignification. Among the 34 laccases, *CcaLAC28* and *CcaLAC32* emerged as potential key players in stem lignification based on their sequence homology with Arabidopsis lignin-modifying laccases. There was a steady expression increase with jute plant development, but significantly lower expression in the mutant jute line *dlpf*, which has 50% less lignin. Future functional validation of these *CcaLAC* genes could help towards improved fibre development and fulfil the industrial demand for low-lignin jute fibre for diversified biodegradable products.

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CRediT authorship contribution statement

Subhadarshini Parida: Formal analysis, Investigation, Methodology. **Deepak Kumar Jha:** Formal analysis, Investigation, Methodology. **Khushbu Kumari:** Formal analysis, Investigation, Methodology. **Seema Pradhan:** Formal analysis, Data curation, Writing - review & editing. **Nrisingha Dey:** Supervision, Writing - review & editing. **Shuvobrata Majumder:** Conceptualization, Investigation, Formal analysis, Funding acquisition, Methodology. Writing – original draft.

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