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

Background: The important industrial bast fibre crop jute (*Corchorus* sp.) is renowned for its long lignocellulosic multi-utility fibres. Information on jute lignin pathway, fibre lignification and related enzymes remains limited. One such key enzyme integral to the final lignification steps is laccase (EC 1.10.3.2).

Results: A whole-genome search of white jute (*Corchorus capsularis*) revealed 34 putative laccase (*CcaLAC*) genes. Phylogenetic analysis categorized these genes into six groups, 17 predominantly expressed in phloem tissue, 9 in leaf, and 4 in xylem and roots. The expression of several *CcaLAC*s increased steadily from plantlet to crop harvest stage. Homology-based selection with *Arabidopsis* laccase (*AtLAC*s), shortlisted *CcaLAC*s involved in lignin polymerisation. Transcriptomics data confirmed their phloem specific expression, with some showing significantly lower expression in *dlpf*, a low-lignin fibre-containing white jute mutant. *CcaLAC* expression also responded to abiotic stresses like ABA hormone and copper heavy metal. Additionally, Ath-miR397a and Ath-miR397b target sites were predicted in 11 and 9 *CcaLAC*s, respectively, suggesting microRNA-mediated post-transcriptional regulation. Subcellular localization showed *CcaLAC*s occupying multiple plant cell compartments. Protein structure predictions revealed up to 10 motifs in CcaLACs, with 18 containing transmembrane

helices. Gene ontology (GO) term analysis linked *CcaLACs* to lignin and phenylpropanoid biosynthesis.

Conclusions: Based on expression patterns under different test conditions *CcaLAC28* and *CcaLAC32* are strong candidates for phloem (bast) fiber lignification process in white jute. Targeted modification of these genes could enhance our understanding of lignification and facilitate the development of low-lignin jute fibres for high-value industrial applications.

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

1. Introduction:

Laccases (LAC) are copper containing enzymes with oxidative efficiency that naturally occur in a diversity of organisms, including many flora [**1**]. They are glycoproteins that can exist as monomers, dimers, or tetramers. These enzymes initiate biodegradation through their oxidation-facilitating catalytic effects on a diversity of organic substrates, while simultaneously reducing molecular oxygen to water [**2**]. There are many studies that have acknowledged the important role of lignin polymerization, played by laccases [**3, 4, 5**]. 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 12-26% lignin content [**6**], which 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 contains no lignin [**7**]. Lowering the lignin content of jute fibres could increase their commercial market value and make their applications versatile.

Research in plant molecular biology, focused at lowering the lignin of natural fibres, requires a deep and wide knowledge of the jute lignification process along with the genes involved. The initial step in this challenging objective is to recognise the relevant gene families

in-silico and within the plant genome. The recently accessible jute reference genome has made the work feasible. The genome size of white jute is 336 Mb, containing 25,874 genes, while the genome size of the tossa jute is 361 Mb with 28,479 genes [**8**]. There are six members in the gene family of Caffeoyl-CoA 3-O-methyltransferase (*CCoAOMT*) that were identified through *in-silico* studies, involved in jute lignification [**9]**. Laccase (*LAC*) and peroxidase (*PER*) contribute to monolignol polymerization that culminates in the lignification process [**10**]. Studies suggest that laccases assist peroxidases during this final step. Specifically, laccases actively participate during the preliminary stages while peroxidases are suggested to be more active during the subsequent developmental phases of the stem [**4**]. In the studies conducted to understand the lignification process the peroxidases received more attention compared to laccases [**11**]. The *LAC/PER* gene families are much unexplored in the case of jute, although they have been studied in many other plants.

In-silico analysis of a genome-wide search for laccase has identified 17 putative *LAC* genes in *Arabidopsis thaliana* [**12**], 30 in rice (*Oryza sativa*) [**13**], 49 in black cottonwood (*Populus trichocarpa*) [**14**], 54 in flooded gum (*Eucalyptus grandis)* [**15**], 45 in flax (*Linum usitatissimum*) [**16**], 84 in cotton (*Gossypium hirsutum*) [**17**], 22 in maize (*Zea mays*) [**18**], and 156 in wheat (*Triticum aestivum*) [**19**]. The presence of multiple *LAC* genes suggest that the encoding for laccases in plants is done by multigene families of size ranging from medium to large. A functional validation study conducted on *Arabidopsis* confirms this. The study involved gene knockout experiments and demonstrated that among the 17 *AtLAC* genes, *AtLAC4* and *AtLAC17* participate in the lignification process [**20**]. Genetic engineering was conducted on poplar by inserting and overexpressing the cotton *GaLAC1* gene in it. This resulted in a 13-fold increase in laccase activity along with 2-19% more lignin [**21**]. 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 of genes involved in the lignification pathway often begins with tissue-specific expression analysis of gene family members. Since stems predominantly contain lignified tissues, it is hypothesized that stem-specific laccases may play a critical role in the lignification process. In *Arabidopsis*, *AtLAC2*, *AtLAC4*, *AtLAC10*, *AtLAC11*, *AtLAC16*, and *AtLAC17* exhibit significant expression specifically in stem tissues, while showing minimal or no

expression in seeds and roots [**20**]. Although, studies involving quadruple and quintuple loss-of-function mutants of *AtLAC4*, *AtLAC5*, *AtLAC10*, *AtLAC12*, and *AtLAC17* revealed that the lignification process is highly specific and depends on distinct cell types and their unique cell wall layers [**22**]. Furthermore, *AtLAC4* and *AtLAC17* were identified as being directly associated with the lignification process [**2, 20**]. To gain a better understanding of the lignification process in jute fibers, it is crucial to focus on the phloem (bast) tissues of the jute stem.

Mutant cultivars prove to be immensely useful in the functional validation of genes. For model plants like *A. thaliana*, *LAC* gene-specific knockout mutants are available for study [**20, 22**]. 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 [**23**]. Transcriptomic analysis of the *dlpf* mutant revealed that multiple genes of the phenylpropanoid and lignin pathways showed lower expression in comparison to its control [**24**]. Further research involving a comparative gene expression study of the *CcaLAC* in *dlpf* and JRC212, throughout their developmental stages, is sure to provide valuable insights into their involvement in the lignification process.

Previous studies suggest that laccases are involved in the management of environmental stress in plants by influencing lignin accumulation or its oxidative functions [**25**]. For example, environmental stress, such as copper heavy metal stress in rice crops, significantly enhances laccase activity and lignin deposition in roots [**13, 26**]. Additionally, in response to multiple environmental stresses, the active involvement of the abscisic acid (ABA) hormone—often referred to as the "stress hormone"—has been reported [**27, 28**]. However, whether laccase expression is induced by ABA hormonal treatment, is yet to be uncovered. If some members of the laccase gene family are responsive to ABA treatment, it is highly likely that they are also involved in other ABA-mediated stress responses, such as desiccation, freezing, and high salinity in plants [**28, 29**]. In the case of jute, no studies have yet reported stress-induced expression of laccase genes. Such studies could provide valuable insights for better prediction of gene function and their association within the laccase gene family.

The *in-silico* study of upstream cis-acting elements of genes provides insights into the stress conditions or signalling pathways that regulate their expression. For instance, stress-

responsive cis elements include STRE, W-box, MYB, MYC, and WUN-motif, whereas elements such as ABRE, ERE, P-box, GARE-motif, and TGA are associated with hormonal signalling. Additionally, elements like G-box, GT1, RY-element, Sp1, GATA-motif, LAMP element, Box-4, and TCT-motif are known to play roles in tissue specificity and developmental processes in plants [**30, 31**]. Post-transcriptional regulation is also a common mechanism in plants, with microRNAs (miRNAs) serving as major regulators of gene expression. *In-silico* studies can predict potential target sites of miRNAs on specific genes, which helps elucidate their possible roles. For example, microRNA397 (miR397), which is conserved across dicots, monocots, and gymnosperms, directly targets laccase transcripts and modulates their expression [**32**]. Therefore, *in-silico* analysis of cis-acting elements and miRNA target sites can provide valuable predictions about the potential functions of genes. This approach is also applicable to the *CcaLAC* gene family, aiding in the identification of their roles in stress responses, signalling, and developmental processes.

Our objective is to identify all *CcaLAC* genes in white jute and analyse their expression levels across various tissues at different developmental stages, as well as under copper stress and ABA treatment. This approach will help us pinpoint the *LAC* genes involved in the lignification process, enabling further functional studies.

2. Materials and Methods

2.1. Identification of Laccase in White Jute Genome and Sequence Analysis

The white jute (*C. capsularis*) genome was retrieved 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. [8]**. Files containing DNA sequences, protein-coding sequences (CDS), and protein sequences were retrieved for *in-silico* analysis.

Laccase protein sequences were retrieved from Oryzabase for *Oryza sativa* [**33**], TAIR for *A. thaliana* [**34**], and the Phytozome database for *Theobroma cacao* [**35**]. From these three plants, as many as 74 *LAC* sequences were used as references to identify *CcaLAC* genes in white jute. The HMMER tool (version 3.2) was used to generate a Hidden Markov Model profile and a

search was conducted against the pre-downloaded jute protein dataset for the purpose of identifying *CcaLAC* genes [**36**].

Confirmation of the presence of the domains that were specific to the known laccase protein family (PF07731.11, PF00394.19, and PF07732.12) in the putative CcaLAC sequences was achieved using NCBI CDD [**37**], HMM Scan [**36**], and SMART [**38**]. The analysis was designed after removing the sequence hits with redundancy cut-off values greater than 0.01, leaving all non-redundant hits [**31**]. The Expasy server ProtParam tool was applied for the analysis of physical and chemical properties of the CcaLAC proteins [**39**]. The CcaLAC proteins have a transmembrane nature and the TMHMM-2.0 [**40**] tool proved vital in assessing it.

2.2. Chromosome Mapping, Phylogeny and Motif Analysis

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

Sequences of the CcaLAC protein from *O. sativa, A. thaliana,* and *T. cacao* and white jute (*C. capsularis*) were used for phylogenetic analysis. For the phylogenetic analysis, *Arabidopsis* was selected as a model plant, rice was included due to its status as a monocot with distinct characteristics from jute, and cacao was chosen because of its high genomic similarity to the jute genome, as reported by **Sarkar et al. [41]** and **Zhang et al. [8]**. The initial software used for sequence alignment was MUSCLE [**42**], followed by MEGA version 10.0 [**43**]. Applying the maximum likelihood method, with 1000 bootstrap replications, gap deletions, and site-gamma (G) distribution rate as parameters, finally the phylogenetic trees were derived [**31**].

The conserved motifs in CcaLAC proteins were analysed using the Multiple Expectation Maximizations for Motif Elicitation (MEME) suite program [**44**]. For predicting conserved motifs, the parameters were set at 10 for maximum number of motifs, minimum width at 6, and maximum width set at 50 [**45**].

2.3. Gene Structure, Gene Duplication and Synteny Analysis

The exon-intron architecture of each *CcaLAC* gene was analysed using the Gene Structure Display Server (GSDS) tool [**46**]. Additionally, gene mutations and duplication events within the *CcaLAC* gene family were examined. To investigate gene duplication patterns, a BLASTp search was conducted, comparing *CcaLAC* proteins with laccase (LAC) proteins from *A. thaliana*, *O. sativa* (rice), *T. cacao* (cacao), and white jute, using an e-value threshold of 0.01. Gene duplications were identified based on sequence similarity of ≥80% [**47**].

To further explore evolutionary divergence, synonymous (Ks) and nonsynonymous (Ka) substitution rates were estimated using the PAL2NAL program [**48**]. Additionally, synteny relationships among the genes were analysed and visualized using TBtools software [**49**].

2.4. Protein-Protein Interaction and Structural Prediction of Candidate Laccase Proteins

To predict protein-protein interaction networks of the candidate laccase proteins (*CcaLAC28* and *CcaLAC32*), we utilized the STRING database (https://string-db.org/, accessed on April 2, 2025) [**50**]. The orthologous *A. thaliana* proteins, AtLAC4 (CcaLAC28) and AtLAC17 (CcaLAC32), were used as references to infer functional protein interactions.

The secondary and tertiary structural features of CcaLAC28 and CcaLAC32 were predicted using the Phyre2 web portal (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, accessed on April 1, 2025). The resulting 3D structures were visualized and analysed using ChimeraX software [**51**].

2.5. Cis-element and microRNA (miRNA) Target Findings on *CcaLAC*

CcaLAC gene upstream regions (≈2000 bp) were extracted from the *C. capsularis* genome, for cis-element analysis. The regulatory cis-elements were identified using the PlantCARE database [**52**]. The analysis focused on identifying cis-elements associated with biotic, abiotic stress

responses, cell cycle regulation, cell wall deposition, circadian rhythms, growth and development, physiology, metabolism, and hormonal signalling.

Arabidopsis mature micro-RNA sequences were retrieved from PmiREN2.0 [**53**]. 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 of white jute [**54**].

2.6. Gene Ontology (GO) Term Assignment

Gene Ontology (GO) terms were assigned to the *CcaLAC* gene family to learn about their functional roles. The protein sequences of CcaLAC were compared against the UniProt-SwissProt database (https://www.uniprot.org/uniprotkb?query=reviewed:true, accessed on January 12, 2025) using BLASTp to identify homologous proteins. The corresponding UniProt IDs were then submitted to the Gene Ontology resource (https://geneontology.org/, accessed on January 15, 2025) for GO term analysis, using *AtLAC* as a reference for annotation.

2.7. Plant Material and Abiotic Stress Treatments

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 for this study from the Central Research Institute for Jute and Allied Fibres (CRIJAF), India. 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. The temperature for sample storage was selected at -80°C to be used for RNA isolation.

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, adhering to the methodology described by **Parida et al. [55]**. Stem tissue sample collection time points were set at six intervals: 0 h, 2 h, 4 h, 8 h, 12 h, and 24 h. Three individual plants were utilised as biological replicates for each time point.

2.8. *In-silico* analysis of *LAC* gene expression

The short reads, generated from the bast (phloem) tissue of control JRC212 and mutant *dlpf* genotypes of white jute, were mapped, with reference to the CDS (coding sequence) sequences of the 34 *CcaLAC* genes. The raw reads for both these samples were generated using the NCBI Sequence Read Archive (Accession number SRR1585372 for JRC212 and Accession number SRR1585373 for mutant *dlpf*). Transcript quantification for the laccase CDS was carried with the help of the scripts align_and_estimate_abundance.pl and a matrix was generated using the script abundance_estimates_to_matrix.pl, from 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 using the MeV software (https://sourceforge.net/projects/mev-tm4, retrieved July 10, 2024).

2.9. Gene Expression Analysis in qRT-PCR

Tissues from different parts of the jute plants (leaf, xylem, phloem, and root) were collected and the total RNA was isolated from them using a commercial RNA isolation kit (Macherey-Nagel, Germany). Adhering to the kit provided protocol, 100 mg of the tissue sample was pulverised in a mortar and pestle using liquid nitrogen. 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, after which, concentration of RNA was estimated with the help of a NanoDrop-2000c spectrophotometer (ThermoScientific, USA). Using a first-strand cDNA synthesis kit (ThermoScientific, USA), first-strand cDNA was synthesised from 1 µg of RNA, following the kit provided protocol.

Primer designing for PCR was done using the PrimerQuest tool 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 list of primers is provided in the **Supplementary Table 1**.

The QuantStudio™ 5 system (ThermoScientific, USA) furnished with QuantStudio™ Design and Analysis Software (V 1.5.2) was used to conduct the qRT-PCR analysis. Freshly synthesise cDNA was diluted 1:9 with molecular-grade water before being used as a sample in qRT-PCR. The conditions for experimental PCR cycle were: initial denaturation at 95 □ for 2 minutes, followed by 40 cycles (15 seconds each) at 95 □, then annealing at the primer-specific temperature (as listed in **Supplementary Table 1**) for 1 minute, and finally extension at 95 □ for 15 seconds. The melt curve of the amplified PCR products was obtained from 60 □ to 95 □. The jute ubiquitin1 (*UBI*) gene (GenBank accession GH985256) was kept as internal control [**55**]. Estimates of the mRNA transcript expression levels were made and represented by relative quantification (RQ) values [**56**]. There were three biological replicates used in total and for each such biological replicate three technical replicates of qRT-PCR analysis were performed.

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 [**2**]. Keeping these *AtLAC* genes as queries in a BLAST search, homologous genes in the jute genome were identified using the TBtools software. *CcaLAC* genes that showed greater than 60% identity with the *AtLAC* sequences were considered homologous and selected for further qRT-PCR based expression analysis [**31**]. Homologous *CcaLAC* gene expression levels were estimated in phloem and xylem tissues at regular time intervals (30 DAS, 60 DAS, 90 DAS, and 120 DAS) of growth to understand the lignification process in the white jute plants.

2.10. Statistical Analysis

A comprehensive analysis was done that included calculation of the mean Ct values using Microsoft Excel and performing a one-way ANOVA. In addition, the GraphPad Prism statistical software (version 9.0) was used to perform Tukey's multiple comparisons test.

3. Results

3.1. Gene Number, Structure, and Duplication Events in White Jute Laccase Genes (CcaLACs)

A total of 34 laccase proteins were discovered in the white jute genome, named *CcaLAC1* to *CcaLAC34* based on their coordinates. These were found to be distributed unevenly across the seven chromosomes in the genome (**Fig. 1A**). Chromosome 7 harboured the highest number of 10 *LAC* genes (*CcaLAC25* to *CcaLAC34*) located on it, followed by chromosome 1 with 7 *LAC* genes (*CcaLAC1* to *CcaLAC7*), and closely followed by chromosome 2 with 6 *LAC* genes (*CcaLAC8* to *CcaLAC13*). Three *LAC* genes were observed on chromosomes 4, 5, and 6 each, while only two *LAC* genes resided on chromosome 3.

The identified *CcaLAC* genes had large variations in exon-intron distribution, ranging from 2-28 numbers of exons. Two exons were present in *CcaLAC19*, whereas the *CcaLAC26* gene held the highest number (28) of exons (**Fig. 1B**).

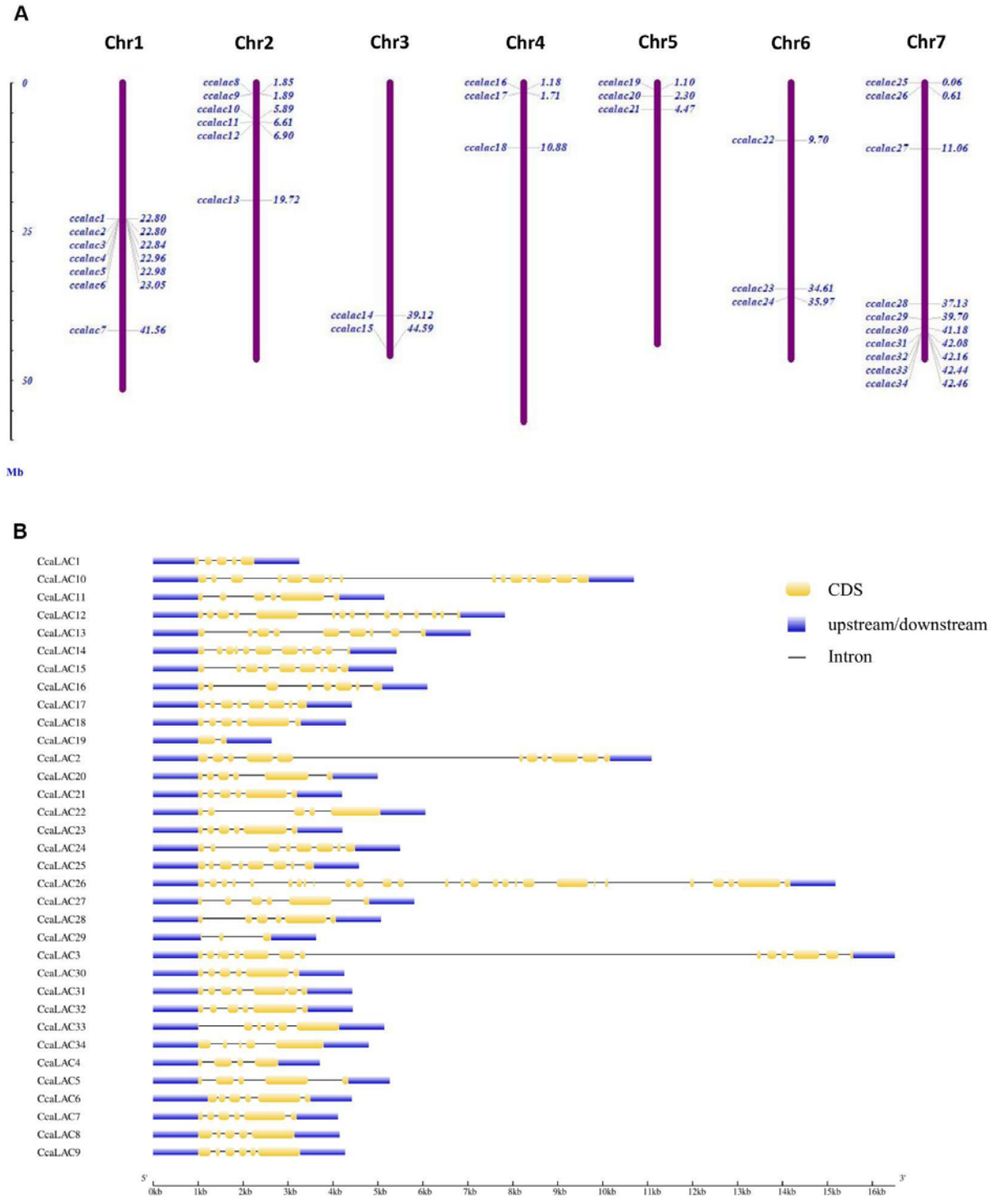


Fig. 1 Number and gene structure of white jute (*C. capsularis*) laccase genes (*CcaLAC*s) (A) Chromosomal mapping of 34 laccase (*CcaLAC*) members in the genome. (B) Gene structure of CcaLACs.

We identified 20 paralogous pairs from gene duplication analysis, comprising 4 tandem pairs and 16 segmental pairs (**Supplementary Table 2**). Additionally, 96 orthologous pairs were identified, which included 21 pairs between *C. capsularis* and *O. sativa*, 21 pairs between *C. capsularis* and *A. thaliana*, and 54 pairs between *C. capsularis* and *T. cacao* (**Fig. 2**). 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.** [**41**] and **Zhang et al.** [**8**].

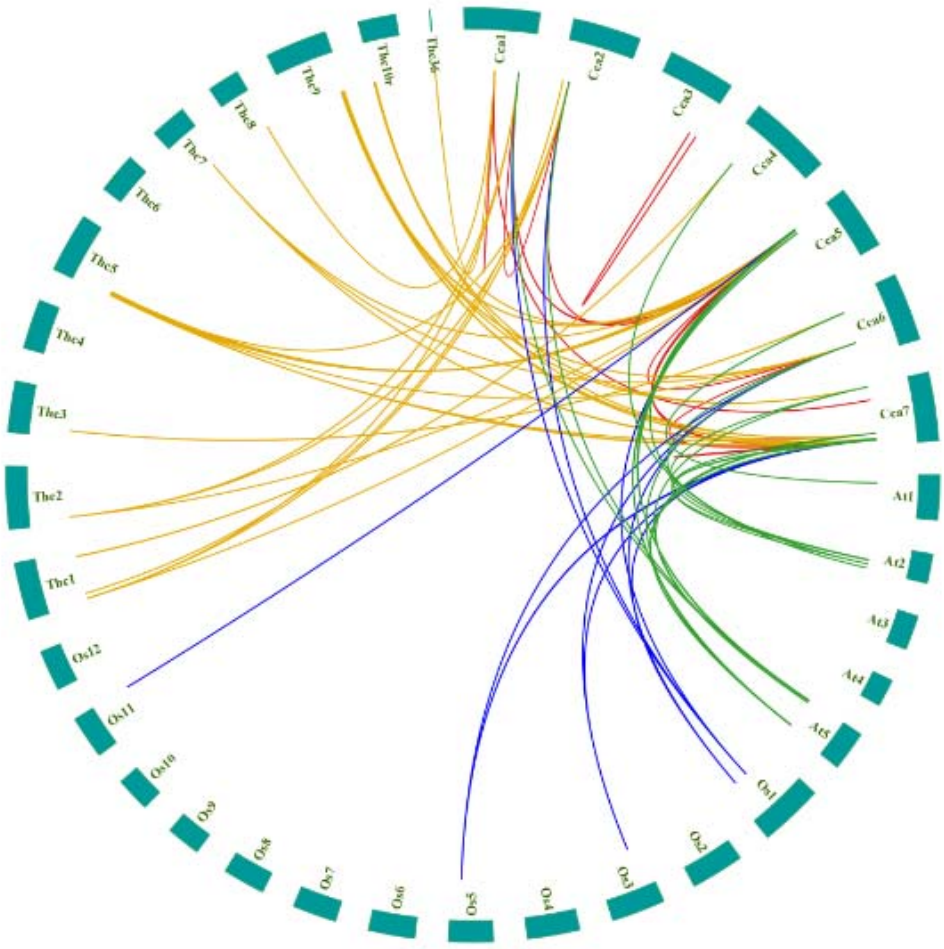


Fig. 2 Synteny relationship between white jute (*C. capsularis*), Arabidopsis (*A. thaliana*), rice (*O. sativa*), and cacao beans (*T. cacao*) *LAC* genes. Each coloured line represents duplicated gene pairs indicating the shared ancestry among the species.

3.2. Motif Analysis and Phylogenetic Relationships of Laccase Proteins in White Jute

The CcaLAC proteins found in white jute varied in length, ranging from 11.65 kDa (CcaLAC29) to 177.31 kDa (CcaLAC26). These proteins are made up of approximately 610 amino acids on an average. The highest number of amino acids found in a CcaLAC protein was 1583, the lowest was 102 (**Table 1**). The instability index of the identified LAC proteins was predicted to be less than 40, except for CcaLAC19 (41.63) and CcaLAC26 (43.34), which is an indication of them being mostly stable in nature (**Supplementary Table 3**). The aliphatic amino acid index of CcaLAC29 (65.1) was the lowest, while CcaLAC26 (93.14) was the highest. The average hydropathy value for all the CcaLACs was less than zero (<0), indicating their hydrophilic nature [**57**]. The identified CcaLAC proteins were predicted to be localised in various organelles of white jute (**Supplementary Table 3**). It was predicted that 52.94% of the CcaLACs were transmembrane proteins in nature, as found in 18 of the total 34 white jute LAC proteins (**Supplementary Figure 1**).

The identified laccase proteins were predicted to comprise 10 motifs, assigned as motif-1 to motif-10 (**Fig. 3**). Almost all of the motifs were present in the majority of the LAC proteins, except for motif-3 and motif-6. CcaLAC19 and CcaLAC29 contained only motif-3 and motif-1, respectively. It was noted that CcaLAC2, CcaLAC3, and CcaLAC10 laccase proteins showed repetitions of certain motifs.

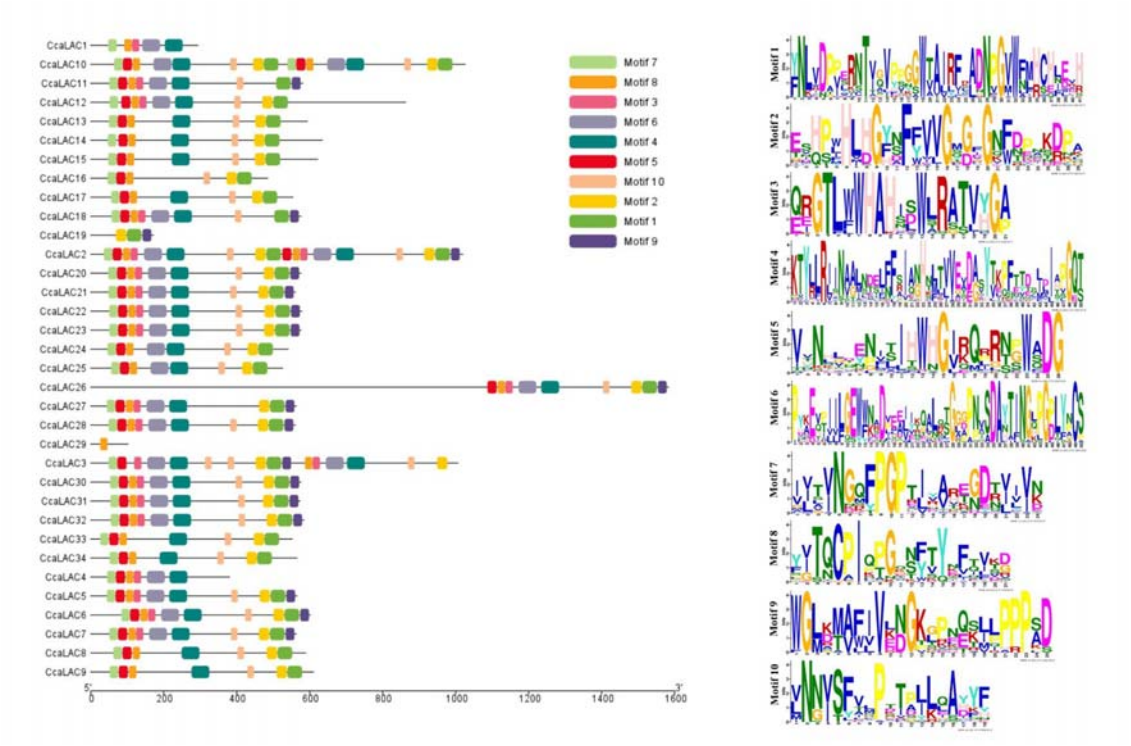
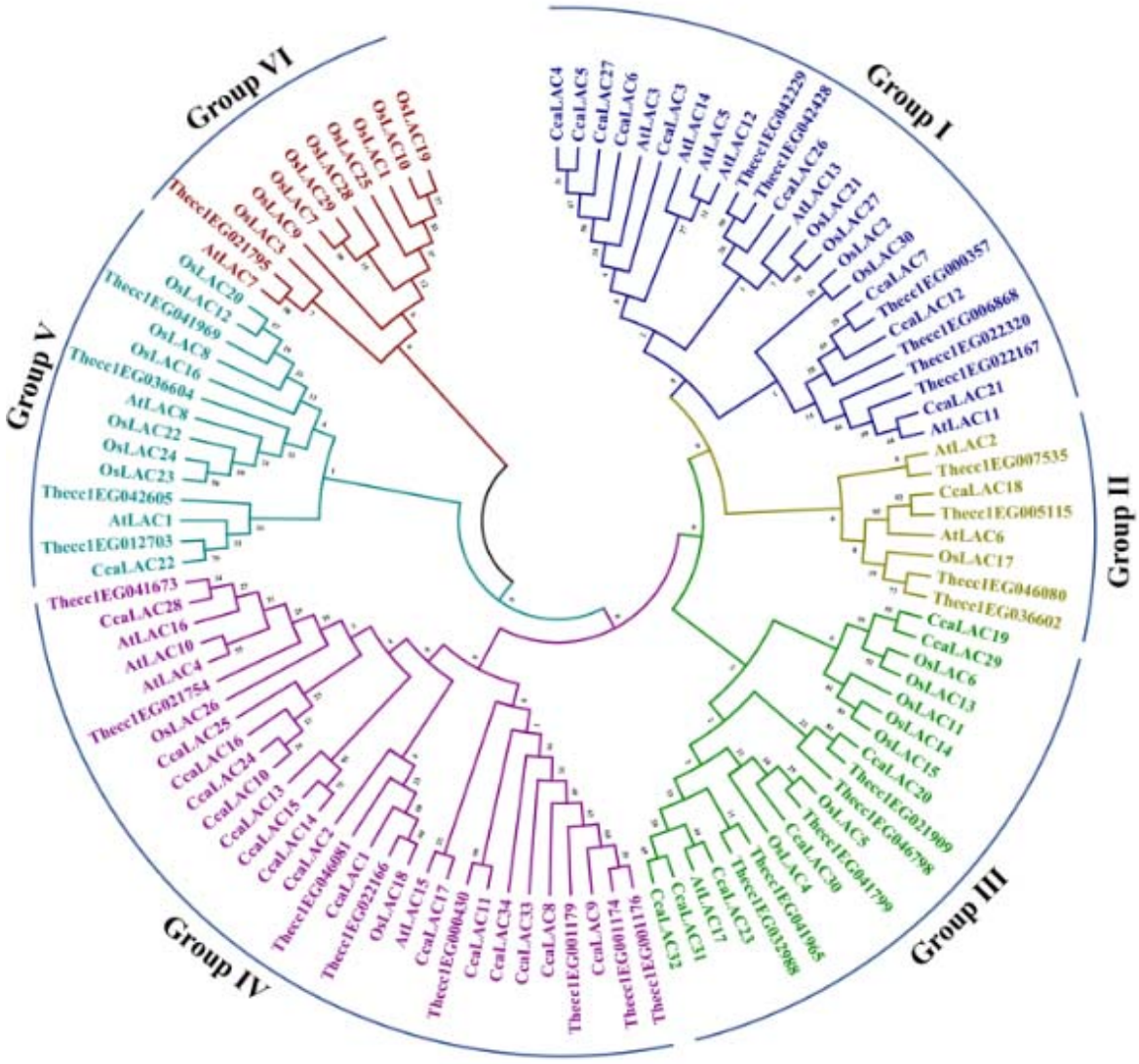


Fig. 3 Conserved motif distribution in identified CcaLAC proteins. Motifs are indicated by different colours and their respective sequences.

The identified laccase proteins of white jute were organised into six groups after phylogeny analysis and labeled as Group I to Group VI (**Fig. 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.

AtLAC4, which is known for its role in the lignification process, clusters in Group IV along with its homologous sequence *CcaLAC28* in jute. Similarly, *AtLAC17* clusters in Group III with its jute homologous sequence, *CcaLAC32* (**Fig. 4**).



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345 **Fig. 4** Phylogenetic tree of LAC proteins of white jute (*Corchorus capsularis*), rice (*Oryza*
346 *sativa*), *Arabidopsis thaliana*, and *Theobroma cacao*. The analysis classified the LAC protein
347 family members into six distinct groups. Abbreviations such as At (*Arabidopsis*), Os (rice),
348 Thecc (*cacao*), and Cca (white jute) are used to denote laccase genes specific to each species.

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352 **Table 1. Bioinformatics analysis and physicochemical properties of C*caLAC*s.**

Gene Name	Gene ID	Gene Length (bp)	Position	Amino Acid (aa)	pI	MW (kDa)	Subcellular Localization
CcaLAC1	GWHPBCLC001173	1330	Chr1	293	5.01	31.85	Chloroplast
CcaLAC2	GWHPBCLC001174	9172	Chr1	1020	6.03	113.52	Chloroplast
CcaLAC3	GWHPBCLC001176	14584	Chr1	1007	5.07	111.63	Endoplasmic Reticulum
CcaLAC4	GWHPBCLC001178	1789	Chr1	381	8.18	42.81	Cytoplasm
CcaLAC5	GWHPBCLC001179	3342	Chr1	566	7.31	63.59	Chloroplast
CcaLAC6	GWHPBCLC001182	2425	Chr1	603	8.64	67.82	Chloroplast
CcaLAC7	GWHPBCLC002591	2192	Chr1	563	8.45	62.29	Cytoplasm
CcaLAC8	GWHPBCLC003786	2149	Chr2	588	8.69	66.19	Mitochondria
CcaLAC9	GWHPBCLC003791	2275	Chr2	611	6.55	68.30	Cytoplasm
CcaLAC10	GWHPBCLC004194	8705	Chr2	1027	9.54	114.71	Cytoplasm
CcaLAC11	GWHPBCLC004256	3145	Chr2	580	7.27	64.94	Vacuoles
CcaLAC12	GWHPBCLC004295	5830	Chr2	862	8.85	95.54	Cytoplasm; Nucleus
CcaLAC13	GWHPBCLC005147	5065	Chr2	594	6.07	66.39	Vacuoles
CcaLAC14	GWHPBCLC008300	3416	Chr3	635	8.72	71.13	Golgi apparatus; Plasma Membrane
CcaLAC15	GWHPBCLC008874	3344	Chr3	622	6.92	69.90	Extracellular Space
CcaLAC16	GWHPBCLC009134	4102	Chr4	485	9.04	54.01	Golgi Apparatus
CcaLAC17	GWHPBCLC009197	2419	Chr4	555	9.12	62.23	Golgi Apparatus
CcaLAC18	GWHPBCLC010239	2289	Chr4	575	8.39	63.76	Extracellular Space
CcaLAC19	GWHPBCLC013380	635	Chr5	171	8.59	18.90	Nucleus
CcaLAC20	GWHPBCLC013482	2995	Chr5	576	9.3	63.87	Peroxisome
CcaLAC21	GWHPBCLC013679	2207	Chr5	561	9.37	62.58	Cytoplasm
CcaLAC22	GWHPBCLC016373	4059	Chr6	578	6.83	64.63	Cytoplasm
CcaLAC23	GWHPBCLC017617	2210	Chr6	577	8.86	63.68	Extracellular Space
CcaLAC24	GWHPBCLC017798	3491	Chr6	542	9.51	60.92	Endoplasmic Reticulum
CcaLAC25	GWHPBCLC018843	2576	Chr7	525	6.05	57.92	Chloroplast

CcaLAC26	GWHPBCLC018907	13184	Chr7	1583	6.66	177.31	Nucleus
CcaLAC27	GWHPBCLC019742	3813	Chr7	564	8.94	63.75	Chloroplast
CcaLAC28	GWHPBCLC020872	3070	Chr7	560	9.28	61.46	Vacuoles
CcaLAC29	GWHPBCLC021142	1626	Chr7	102	10.15	11.65	Nucleus
CcaLAC30	GWHPBCLC021267	2250	Chr7	576	9.41	63.62	Vacuoles
CcaLAC31	GWHPBCLC021382	2427	Chr7	574	9.19	63.11	Nucleus
CcaLAC32	GWHPBCLC021391	2443	Chr7	584	9.33	64.46	Nucleus
CcaLAC33	GWHPBCLC021419	3143	Chr7	551	6.36	61.84	Cytoskeletons
CcaLAC34	GWHPBCLC021420	2794	Chr7	565	6.4	63.17	Cytoplasm; Nucleus

3.3. Cis-acting Elements

An assortment of cis-elements were prognosticated in the upstream region of the 34 *CcaLAC* genes (**Supplementary Figure 2**). The MYB, MYC, and TATA-box inhabited them all. The cis-elements linked coherently with various vital functions, including stress (STRE, W-box, MYB, MYC, WUN-motif), hormonal signalling (ABRE, ERE, P-box, GARE-motif, 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 regions upstream of the *CcaLAC* genes, suggesting diversity in their functional abilities [**30, 31**].

The presence of cis-acting elements, related to secondary wall formation, has been identified upstream of multiple *CcaLAC* genes (**Supplementary Table 4**), including AC elements (ACE), G-box motifs, MYB Binding Sites (MBS), and other cis-elements that are not only involved in hormonal signalling but also play significant roles in plant cell wall formation [**58-60**].

3.4. Tissue-Specific Expression Analysis of *CcaLAC* Genes

An expression was observed in the qRT-PCR data for all of the 34 *CcaLAC* genes, derived from four different sample tissues (phloem, xylem, leaf, and root). The mRNA transcript expression

levels are represented by relative quantification (RQ) values, with root tissue expression set as the reference (RQ value 1) (**Fig. 5**). The following observations were made: Expression levels were observed to be higher in leaf tissues than in other tissues 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*.

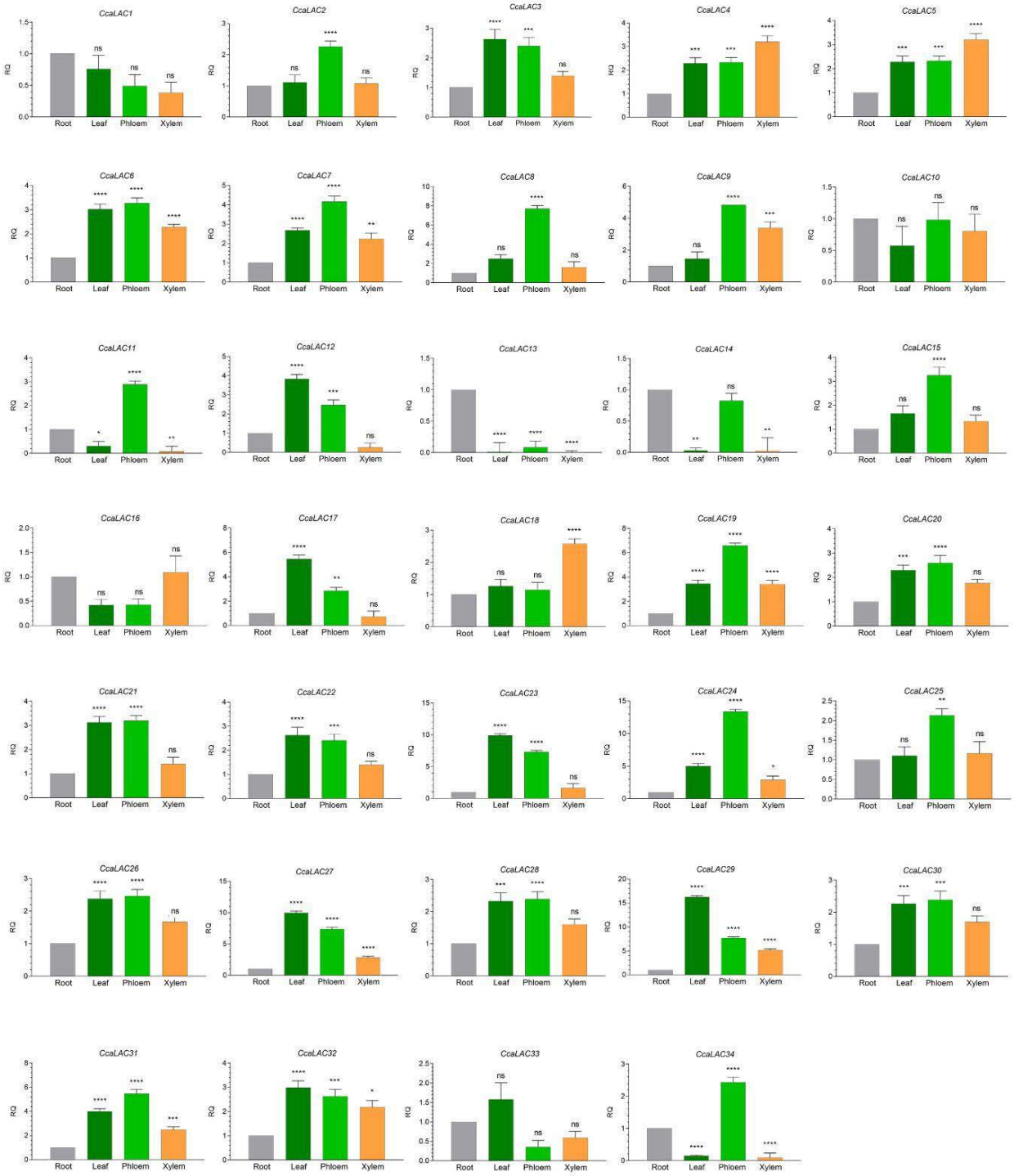


Fig. 5 Tissue-specific expression of laccase (*CcaLAC*) genes. This figure shows the relative quantification (RQ) values of *CcaLAC* gene expression in the tissues of leaf, phloem, xylem, and root. The RQ values are normalised using the ubiquitin 1 (*UBI*) housekeeping gene. A one-way

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.

3.5. Phloem Tissue Specific Expression Analysis of *CcaLAC* Genes Across 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, 90, and 120 DAS (time for harvesting fibre)—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 (**Fig. 6**). *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.

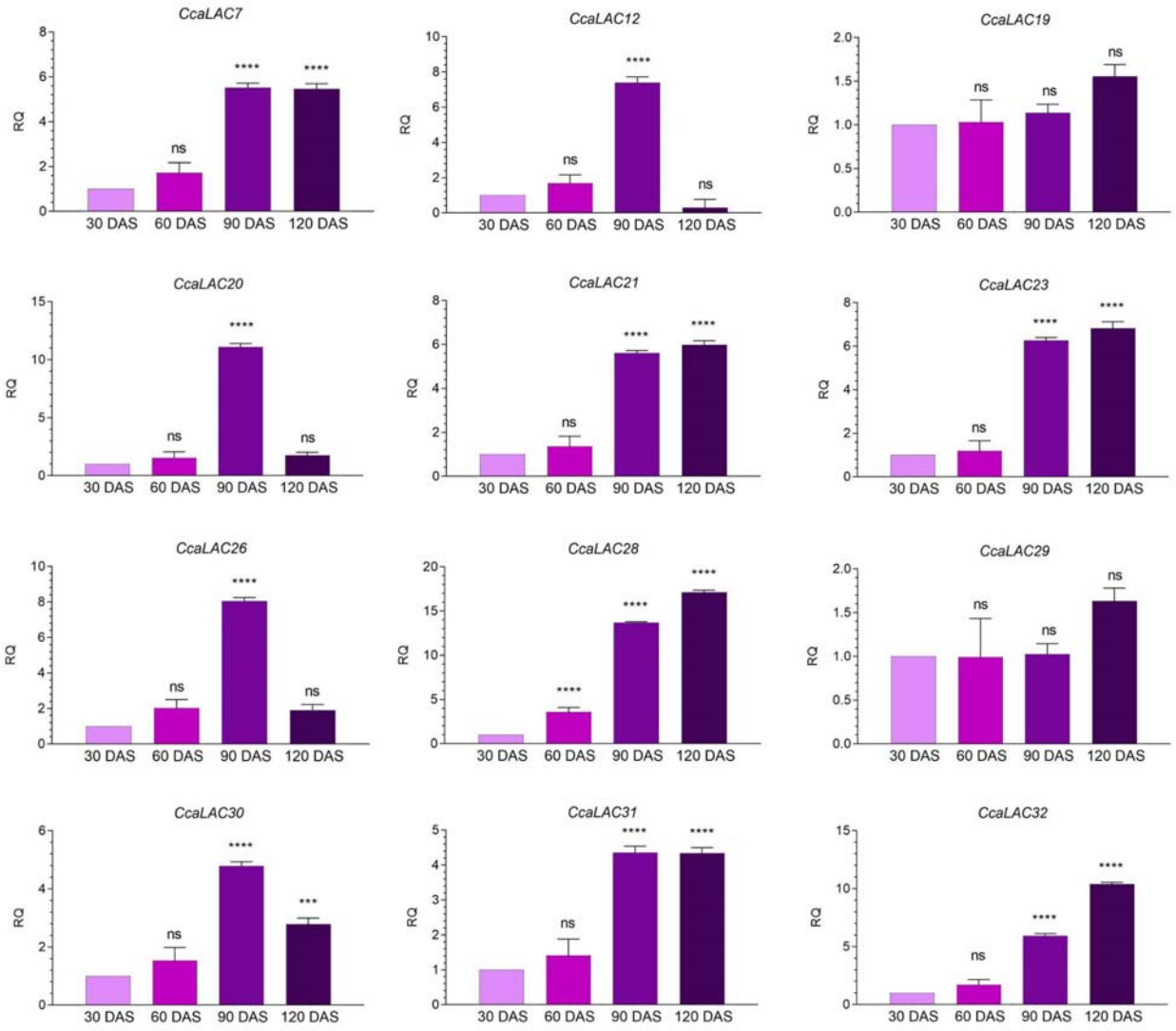


Fig. 6 Expression of laccase (*CcaLAC*) genes at different developmental stages of white jute plants. This figure illustrates the expression of *CcaLAC*s in the phloem tissue of white jute at four different time points, from 30 to 120 days after sowing (DAS). A one-way ANOVA (*F*-test) with a significance 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.

3.6. Expression Analysis of *CcaLAC* Genes in the Low-Lignin Mutant Jute (*dlpf*)

The transcriptomics data from 30 DAS phloem tissue of JRC212 and its X-ray-irradiated mutant line *dlpf,* helped in performing *in-silico* expression analysis of the *CcaLAC* genes. RNAseq raw reads of phloem tissue of JRC212 and *dlpf* were sourced from the study by **Chakraborty et al. [24]**. All the 34 laccase genes were observed to express in the phloem tissue (**Fig. 7**). 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**).

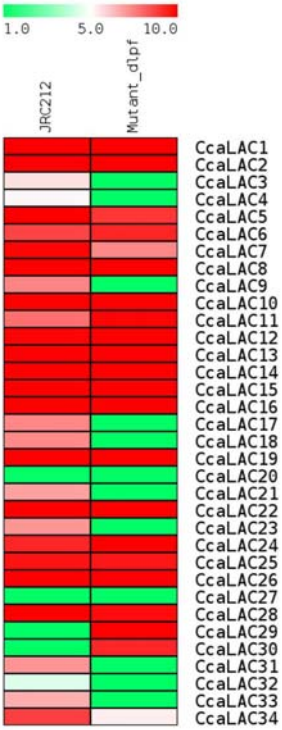
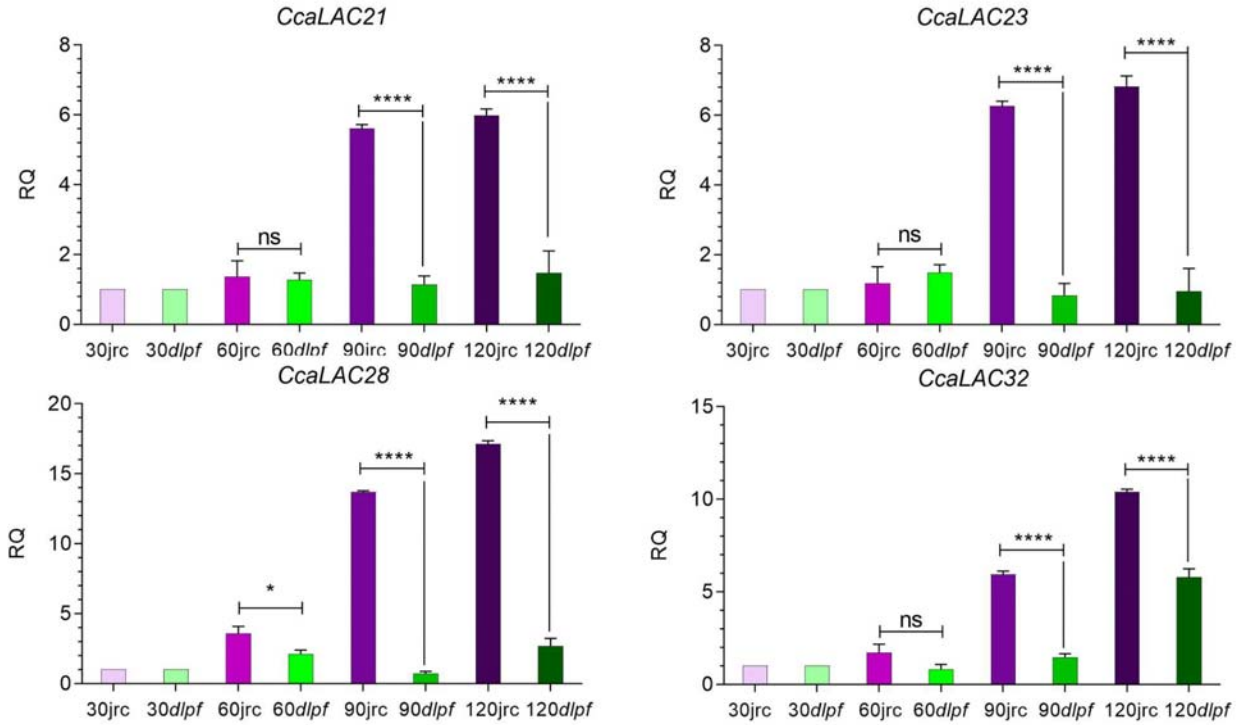


Fig. 7 Expression analysis *in-silico* of laccase genes (*CcaLAC*s) in the phloem tissue of JRC212 (control) and *dlpf* (mutant) at 30 DAS. Heat map is based on publicly available transcriptomics data from BioProject PRJNA255934. The expression scale ranges from low (1, green) to high (10, red).

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. The

426 expression decreased significantly (P<0.0001) in *dlpf* compared to JRC212 at 90 DAS and 120
427 DAS for these four genes (**Fig. 8**). A similar decrease in expression was noted in *dlpf* at 90 DAS
428 for *CcaLAC12*, *CcaLAC20*, and *CcaLAC26* genes. These three genes showed high expression in
429 JRC212 at 90 DAS compared to other time points but did not show such expression in *dlpf*
430 (**Supplementary Figure 4**). Two genes, *CcaLAC19* and *CcaLAC29*, exhibited minimal
431 expression in JRC212 at all time points and nonsignificant differences in expression in *dlpf*
432 compared to JRC212 (**Supplementary Figure 4**). This comparative analysis aims to elucidate
433 the contrast in *CcaLAC* gene expression patterns that was observed between the JRC212 control
434 and the *dlpf* mutant jute.

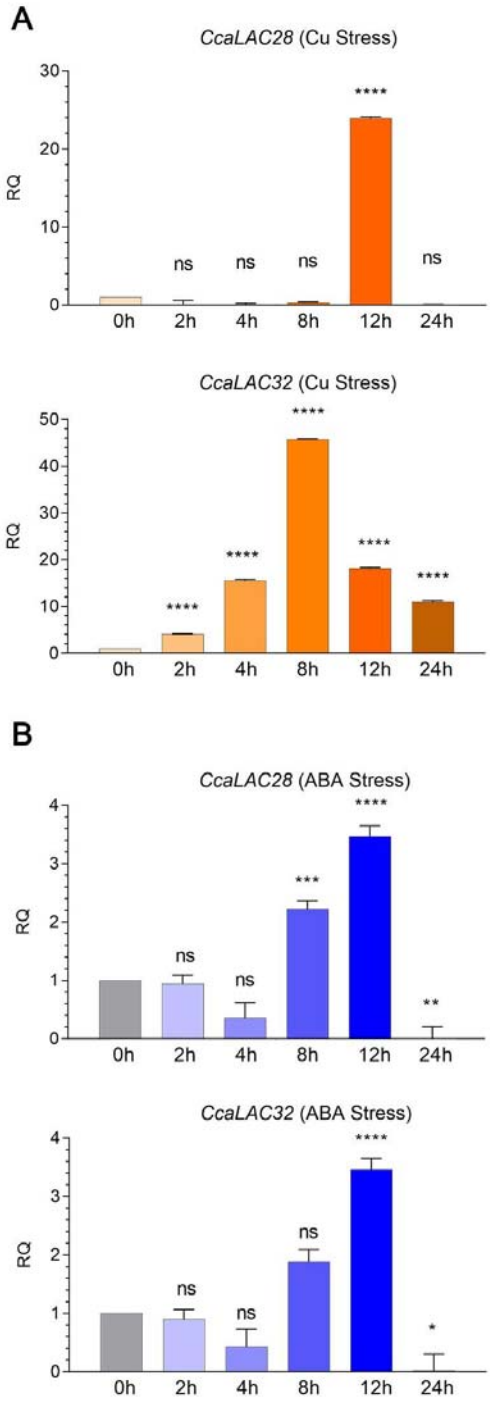


435

436 **Fig. 8** Comparative study of *laccase* (*CcaLAC*) gene expression in white jute cultivar JRC212
437 and its mutant line *dlpf*. This figure presents a comparative expression analysis of laccase
438 (*CcaLAC*) genes of JRC212 and *dlpf* (which has fibres with 50% lower lignin content), across
439 different developmental stages (30 DAYS to 120 DAS). A one-way ANOVA (F-test) with a
440 significance threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was
441 performed. Asterisks denote significant differences in P values from Tukey's test, while non-
442 significant values are marked as 'ns'. The data represent three biological replicates.

3.7. Expression Analysis of CcaLAC Genes Under Copper Stress and ABA Hormonal Treatment

CcaLAC28 and *CcaLAC32*, are two highly expressive genes that are homologous to *AtLAC4* and *AtLAC17* respectively. As *AtLAC4* and *AtLAC17* are considered vital to the lignin synthesis pathway in Arabidopsis, the expression of their homologous counterparts in white jute needed thorough examination in plants that were subjected to copper heavy metal stress. In *CcaLAC28,* a significant increase in expression was observed at 12 hours, whereas in *CcaLAC32* all the time points (from 2 hours to 24 hours) showed significantly high expression under the copper stress. The *CcaLAC32* expression gradually increased and was highest at 8 hours, then it gradually decreased (**Fig. 9A**).



454

455 **Fig. 9** Expression of *CcaLAC28* and *CcaLAC32* under abiotic stresses. This figure depicts the
456 *CcaLAC28* and *CcaLAC32* genes expression levels in (A) copper stress using (0.10 mM
457 $CuSO_4 \cdot H_2O$), (B) ABA hormone stress. A one-way ANOVA (F-test) with a significance
458 threshold of $P \leq 0.05$, followed by Tukey's multiple comparisons test, was performed. Asterisks

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

461

462 The level of expression by *CcaLAC28* and *CcaLAC32* genes was recorded in plants
463 treated with ABA hormone stress. There was a noteworthy rise in expression observed at 8 h and
464 12 h for both the genes (**Fig. 9B**). The expression level was highest at 12 h and lowest at 24 h.
465 These patterns of *CcaLAC28* and *CcaLAC32* gene expression suggest that they act as delayed
466 responsive genes in white jute under ABA hormonal stress.

467

468 **3.8. Protein Structure Analysis of CcaLAC28 and CcaLAC32**

469 The protein structures of *CcaLAC28* and *CcaLAC32* were analysed using Phyre2.2 [**61**]. The
470 *CcaLAC28* protein, consisting of 531 amino acid residues, was modelled with 100% confidence.
471 It shared 48% sequence identity with the crystal structure of *Zea mays* laccase 3 (ZmLAC3)
472 complexed with sinapyl (fold library ID: c6kliA). The predicted secondary structure composition
473 included 39% β-strands, 4% α-helices, and 7% disordered regions. Notably, a transmembrane
474 domain was predicted between amino acid residues 226 and 241 (**Fig. 10A**).

475 Similarly, *CcaLAC32*, with 540 amino acids, was also modelled with 100% confidence. It
476 exhibited 50% sequence identity with ZmLAC3. The structural analysis revealed that *CcaLAC32*
477 comprised 34% β-strands, 5% α-helices, and 4% disordered regions. Additionally, a
478 transmembrane domain was predicted between amino acid residues 136 and 151 (**Fig. 10B**).

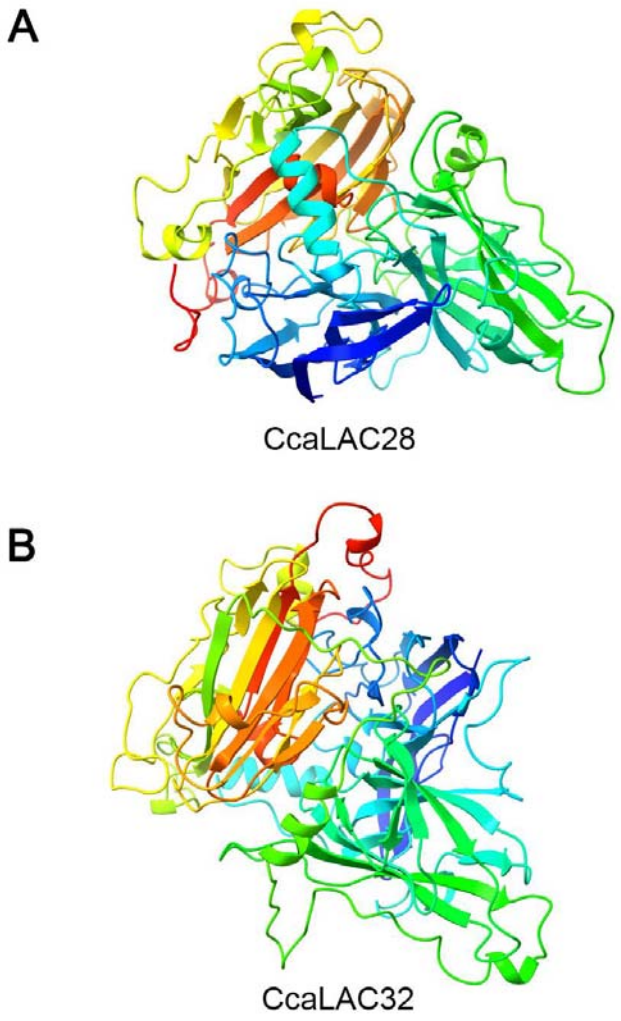


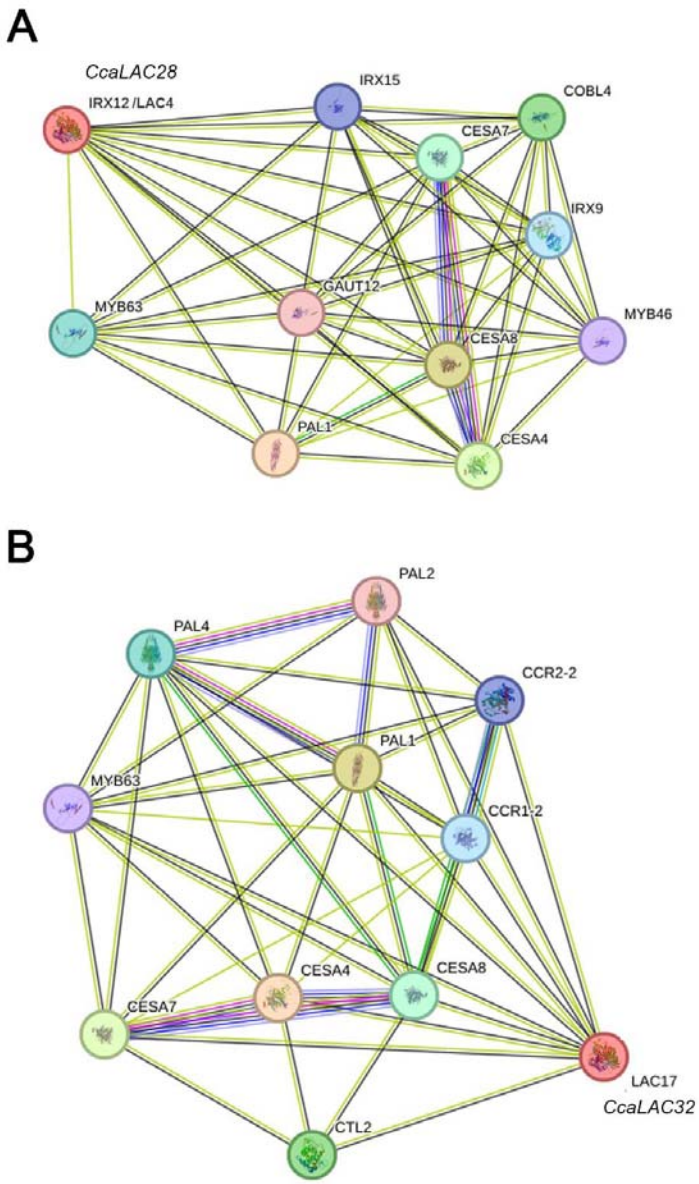
Fig. 10 Predicted three-dimensional protein structures of *CcaLAC28* and *CcaLAC32*. (A) The structural model of *CcaLAC28* (531 amino acids) and (B) *CcaLAC32* (540 amino acids) were generated using Phyre2.2. The structures are color-coded from the N-terminus (blue) to the C-terminus (red), highlighting secondary structural elements such as β-strands, α-helices, and loop regions.

3.9. Protein-Protein Interaction Network Analysis

The protein-protein interaction network was analysed *in-silico* using the STRING database. Since the jute genome is not available in STRING, the *Arabidopsis thaliana* (NCBI Taxonomy ID: 3702) genome was used as a reference to investigate the interaction network of *AtLAC4*,

which is homologous to *CcaLAC28*, and *AtLAC17*, homologous to *CcaLAC32*. The STRING database revealed interactions of *CcaLAC28/AtLAC4* with key enzymes and regulatory proteins involved in lignin biosynthesis and secondary cell wall formation. These interactions include phenylalanine ammonia-lyase (*PAL1*), cellulose synthase A catalytic subunits (*CESA4, CESA7*, *CESA8*), and transcription factors such as *MYB63* (a regulator of lignin biosynthesis) and *MYB46* (a regulator of secondary wall synthesis). Additionally, interactions were observed with *IRX9* (a beta-1,4-xylosyltransferase involved in secondary cell wall synthesis), *IRX15* (required for xylan biosynthesis), *COBRA-like protein 4* (*COBL4*), and *GAUT1* (involved in pectin assembly and distribution) (**Fig. 11A**).

The STRING database revealed that *CcaLAC32/AtLAC17* interacts with *PAL1, PAL2*, *PAL4*, *CESA4*, *CESA7*, *CESA8* and *MYB63*, similar to *CcaLAC28*. However, *CcaLAC32/AtLAC17* also interacts with *cinnamoyl-CoA reductase 1* (*CCR1-2*), which is involved in the lignin biosynthesis pathway, and *CCR2-2*, which plays a role in the plant hypersensitive response. Additionally, it interacts with *chitinase-like protein 2* (*CTL2*), which is involved in cell wall biosynthesis (**Fig. 11B**).



505

506 **Fig. 11** Protein-protein interaction network analysis of CcaLACs using the STRING database.
507 (A) Predicted interaction network of *CcaLAC28* (homologous to *AtLAC4*). (B) Predicted
508 interaction network of *CcaLAC32* (homologous to *AtLAC17*).

509

510 **3.10. MicroRNA (miRNA) Targeting of *CcaLAC* Genes**

511 White jute laccase genes (*CcaLACs*) were predicted to be target sites for commonly known
512 lignin-related miRNAs (**Supplementary Table 5**), 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 Fig. 5**).

3.11. Functional Role Analysis of *CcaLAC* Genes Through Gene Ontology (GO) Term Assignment

GO term assignment revealed that the *CcaLAC* genes are associated with several biological processes, including the lignin biosynthetic process (GO:0009809), lignin metabolic process (GO:0009808), phenylpropanoid biosynthetic process (GO:0009699), phenylpropanoid metabolic process (GO:0009698), secondary metabolite biosynthetic process (GO:0044550), and secondary metabolic process (GO:0019748) (**Supplementary Fig. 6**). Furthermore, the GO analysis indicated that *CcaLAC* genes are involved in various molecular functions, such as hydroquinone:oxygen oxidoreductase activity (GO:0052716), oxidoreductase activity (GO:0016682), copper ion binding (GO:0005507), and transition metal ion binding (GO:0046914) (**Supplementary Fig. 6**). These molecular functions are consistent with those typically associated with the laccase gene family.

4. Discussion

The white jute genome contains 25,874 genes [**8**], including the much unexplored laccase (*CcaLAC*) genes, especially regarding the function in stem development, fibre lignification, and performance under abiotic and biotic stress. The research was aimed at finding the functional importance of *LAC* in white jute (*C. capsularis*). The whole white jute genome was searched and 34 *CcaLAC* genes were identified; their expressions were examined in different tissues (**Fig. 5**). 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

where *AtLAC* genes (*AtLAC2, AtLAC4, AtLAC10, AtLAC11, AtLAC16*, and *AtLAC17*), responsible for the lignification process, were expressed excessively in stem tissue of *Arabidopsis* [**2**]. This tissue-specific expression pattern of *LAC* genes in white jute (*C. capsularis*) differs from that observed in tossa jute (*C. olitorius*). In tossa jute, 43.47% (20 out of 46) of the *ColLAC* genes exhibited higher expression in roots compared to other tested tissues [**62**]. This suggests potential differences in regulatory mechanisms between the two *Corchorus* species that govern the tissue-specific expression patterns of *LAC* genes.

Separation of xylem and phloem tissue from the jute stem is difficult in the initial days of its growth, but by 30 DAS it becomes much easier, suggesting that fibre development and the lignification process begin around this time [**24**]. 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 [**7**]. This research included targeting monthly time points of 30 DAS (one month), 60 DAS (two months), 90 DAS (three months), and finally 120 DAS (4 months) to relate *CcaLAC* expression with jute stem development and fibre lignification process.

As a part of this investigation to find the functional importance 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 [**63**]. There is a remarkable similarity between the sequences of *CcaLAC* and *AtLAC* genes and their expression patterns also match those of *Arabidopsis*, increasing with plant development [**20**] (**Fig. 6**).

Mutation of *Arabidopsis AtLAC4* (homologous to *CcaLAC28*) and *AtLAC17* (homologous to *CcaLAC32*) individually resulted in a 10% reduction in lignin content, and when both genes (*AtLAC4+AtLAC17*) were mutated, lignin content was reduced by 39% [**20**]. 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 [**64**]. 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

have significant contribution in the process of lignification. 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 (**Fig. 6**). This raised the question: Are these genes important in the process of lignin production in jute? Finding the answer led us to examine the expression of these four *CcaLAC* genes in a low lignin fibre content mutant variety of white jute, *dlpf* at various stages of growth. We found statistically significant differences upon comparing the expression patterns of these four *CcaLAC* genes of *dlpf* with that of its control, JRC212. Lower expression patterns of *CcaLAC21*, *CcaLAC23*, *CcaLAC28* and *CcaLAC32* were found, which could be a contributing factor to the low lignin concentration observed in *dlpf* (**Fig. 8**). Although *dlpf* is an X-ray-irradiated mutant and may have mutations in other lignin pathway genes, the differential expression pattern of its control (JRC212) on four *CcaLAC* genes (*CcaLAC21*, *CcaLAC23*, *CcaLAC28*, and *CcaLAC32*) suggests that these genes are potential candidates for further investigation.

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 Fig. 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 Fig. 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 Fig. 4**). This consistent expression suggests a fundamental role played by these genes in white jute development.

Previous studies (up to 60 DAS) on the low-lignin mutant jute, *dlpf*, by **Chakraborty et al. [24]** found a low expression of cinnamyl alcohol dehydrogenase (CAD), considered to be essential the lignin pathway. This recent study on the *dlpf* (plants up to 120 DAS) lignin pathway

involved comparing the qRT-PCR expression data using two housekeeping genes- *UBI* and *beta-tubulin* (*BTUB*) [**55**]. We found the expression of *CCoAoMT*, cinnamate 4-hydroxylase (*C4H*), and *CAD* to be lower in *dlpf* than in its control JRC212. This study represents the first analysis of *LAC* gene expression in the low-lignin mutant *dlpf*. Although transcriptomic data (BioProject PRJNA255934) from Chakraborty et al. (2015) reported 15 unigenes annotated as *LAC* genes in *JRC212* and *dlpf*, no qRT-PCR-based expression analysis was conducted. Here, we utilized the same transcriptomic dataset to examine the *in-silico* expression profiles of all 34 *CcaLAC* genes, revealing distinct expression patterns (**Fig. 7**). The low expression levels 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 (**Fig. 8**).

The expression pattern of plant laccases is influenced by transcriptional and post-transcriptional factors. The MYB transcription factors regulate *LAC*, as reported in multiple plant studies. They are one of the largest transcription factor families that are most functionally diverse. In Arabidopsis, *AtMYB58* directly regulates *AtLAC4* expression; *AtMYB46* regulates *AtLAC10* and *AtHY5* regulates *AtLAC12* [**58**]. In *Phyllostachys edulis*, *PeMYB4.1/20/85.2* regulates *PeLAC20*; in *Pyrus bretschneideri*, *PbMYB26* regulates *PbLAC4*-like; and in *Pennisetum glaucum, PgMYB305* regulates *PgLAC14* [**65**]. A predominance of MYB binding sites were found in many jute laccases (*CcaLAC* genes) after analysis of cis-elements in their upstream sequences (**Supplementary Fig. 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 [**66**].

The role of miRNAs in regulation of LAC has been reported in multiple plants, including rice, Arabidopsis, and Populus. MicroRNA397 (miR397) is directly targeting laccase transcripts and controlling their expression in plants [**32**]. In Populus, 28 *PtLAC* genes are targeted by miR397 [**67**]. In chickpea (*Cicer arietinum*) the *CaLAC4* and *CaLAC17L* genes are targeted by miR397 out of the 20 *CaLAC* genes [**68**]. The expression of *OsLACs* in rice is another regulatory role played by miR397 [**69**]. It is considered as a negative regulator of *LAC* expression in lignin biosynthesis, as reported in rice, chickpea, Arabidopsis, and Populus [**14**].

Are the *CcaLACs* targeted by miR397? We found that miR397a targets 11 *CcaLAC*s, and miR397b targets 9 *CcaLAC*s in white jute (**Supplementary Fig. 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. [70]**, could provide more information on its involvement in the lignification process by regulating *CcaLACs* in the near future.

The assortment of cis-acting elements located in the upstream promoter regions of *CcaLACs* suggests that the expression of *CcaLAC* genes may be influenced by diverse factors, including plant growth, developmental stages, hormonal signals, and both abiotic and biotic stresses (**Supplementary Fig. 2**). Notably, cis-acting elements associated with secondary wall formation, such as AC elements (ACE), G-box motifs, and MYB Binding Sites (MBS), were identified in multiple *CcaLACs* (**Supplementary Table 4**). **Zhou et al. [58]** demonstrated in *Arabidopsis* that *AtMYB58* and *AtMYB63* directly activate lignin biosynthetic genes by targeting ACE. **Zhao et al. [59]** also reported that the lignin-specific transcription factor *AtMYB58* in *Arabidopsis* can directly activate most lignin biosynthetic genes through ACE binding in their promoters. Additionally, G-box motifs or G-box Regulating Factors (GRFs) are known to play crucial roles in plant physiological and developmental processes as well as in stress responses [**60**]. The presence of these cis-acting elements in the *CcaLAC* members highlights their potential involvement in the lignification process and secondary wall formation in jute.

The expression pattern of *CcaLAC28* (homologous to *AtLAC4*) and *CcaLAC32* (homologous to *AtLAC17*) 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., [65]**), 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.

The GO term assignment revealed that the *CcaLAC* genes are associated with the molecular function of copper ion binding (GO:0005507) (**Supplementary Fig. 6**). This suggests that some of the identified 34 *CcaLAC* genes might respond to copper, a hypothesis that was further tested under 0.10 mM $CuSO_4 \cdot H_2O$ stress. Under copper 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 (**Fig. 9A**). Such high expression was also previously recorded in rice laccase 10 (*OsLAC10*) under copper stress, which showed a 1200-fold increase at 12 h [**13**]. Our goal was to identify *CcaLAC* genes involved in the lignification process, and copper stress was applied based on previous reports to identify responsive genes that could potentially be laccases. Further biochemical analysis will be required to confirm their laccase activity.

ABA hormone stress mimics conditions of multiple stresses like desiccation, freezing and high salinity in plants [**29**]. Additionally, literature supports that drought stress can alter laccase expression in plants [**68, 71**]. We found that *CcaLAC28* (homologous to *AtLAC4*) and *CcaLAC32* (homologous to *AtLAC17*) responded to ABA stress (**Fig. 9B**). The presence of cis-acting elements such as MYC, MYB, CAT-box, and W-box which are known for ABA responsiveness in plants, likely explains this expression pattern [**72**]. 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 and functionality, under the influence of 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 3**). 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*. *In-silico* subcellular localization studies for *CcaLACs* provided vital information by predicting its location in cellular organelles, such as the chloroplast, plasma membrane, endoplasmic reticulum, mitochondria, Golgi apparatus, 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

687 to the presence of transmembrane helices in the majority (18 of 34) of *CcaLAC*s, as found in our
688 analysis (**Supplementary Fig. 1**). The structure of the plant laccase is still not well studied.
689 Information about the presence of transmembrane helices could provide valuable insights into its
690 structure.

691 Functional role analysis of *CcaLAC* genes through Gene Ontology (GO) term assignment
692 identified 34 *CcaLAC* genes in white jute as being associated with key biological processes,
693 including the phenylpropanoid biosynthetic (GO:0009699) and metabolic (GO:0009698)
694 pathways (**Supplementary Fig. 6**). Lignin, a crucial structural polymer in plants, is a product of
695 the phenylpropanoid pathway. The GO term analysis further predicted that these *CcaLAC* genes
696 are involved in the lignin biosynthetic (GO:0009809) and metabolic (GO:0009808) processes.
697 While these functional roles are supported by the GO term assignment, experimental validation
698 is required to confirm these predictions. Nevertheless, the identification of these 34 genes
699 strongly suggests their involvement in the lignification process, highlighting their potential
700 significance in white jute.

701 Expression of *LAC* genes, including its up- and down-regulation, has been studied
702 extensively in multiple crops. In cotton, down-regulation of *GhLAC1* increases fibre initials and
703 shortens fibre length [**73**]. Silencing of *TaLAC4*, a wheat *LAC* gene, led to a substantial decrease
704 in the total stem lignin deposition [**74**]. In pear (*Pyrus bretschneideri*), overexpression of
705 *PbLAC1* enhanced lignin production and thereby promoted cell wall development [**75**]. In
706 silvergrass (*Miscanthus* sp.), overexpression of *MsLAC1* led to an increase in total lignin [**76**].
707 Downregulation of *CcaLACs* in white jute could alter its lignin content with or without any
708 physiological changes. To avoid major physiological changes in jute plants due to the alteration
709 of lignin content through downregulation of *CcaLAC*s, phloem-specific silencing of *CcaLAC*s
710 can be possible in jute, using the AtSUC2 phloem specific promoter as described by **Majumder**
711 **et al. [77]**. Transgenic research on jute is gaining momentum. Multiple agronomically important
712 traits like insect resistance (IR) [**7, 78**], fungus resistance (FR) [**79**], herbicide tolerance (HT)
713 [**79**], or a combination of these traits (IR-FR-HT) [**80**] have been developed for white jute
714 (cultivar JRC321). Downregulation of lignin pathway genes (*COMT, C4H, C3H, F5H*) through
715 RNAi has also been reported in tossa jute [**81**]. Further study on the overexpression or
716 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, GO term assignment suggesting their likely involvement in the lignification process. 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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Author's contributions

SP performed all the wet lab experiments. DKJ conducted all the dry lab (bioinformatics) analyses. KK organized the data and prepared the tables. SP also contributed to bioinformatics data interpretation and result analysis. ND analysed the wet lab gene expression data. SM was responsible for experiment design, data analysis of both wet and dry lab experiments, and manuscript writing. All authors reviewed and approved the final manuscript.

Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Fig. 1: Prediction of transmembrane helices in CcaLACs.

Supplementary Fig. 2: Cis-acting elements in the upstream regions of CcaLACs.

Supplementary Fig. 3: Validation of *in-silico* heatmap (transcriptomics) data using qRT-PCR.

Supplementary Fig. 4: Comparative study of laccase (*CcaLAC*) gene expression in white jute cultivar JRC212 and its mutant line dlpf. This figure presents a comparative expression analysis of laccase (*CcaLAC*) genes in JRC212 and *dlpf* (which has fibres with 50% lower lignin content) across different developmental stages (30 DAS to 120 DAS).

Supplementary Fig. 5: MicroRNA target prediction in *CcaLAC*s

Supplementary Fig. 6: Gene Ontology (GO) term classification of the *CcaLAC* gene family.

Supplementary Table 1: List of primers

Supplementary Table 2: List of orthologous and paralogous pairs of *CcaLAC* genes

Supplementary Table 3: Protein features and chromosomal details of identified 34 *CcaLAC*s members in *Corchorus capsularis*

Supplementary Table 4: Functions of identified cis-regulatory elements in *CcaLAC* genes

Supplementary Table 5: miRNA Targets in *CcaLAC*s