Comparative Transcriptomic Analysis Identifies Key Cellulose Synthase Genes (CESA) and Cellulose Synthase-Like Genes (CSL) in Bast Fiber Development Stage of Flax (*Linum Usitatissimum* L.)

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Abstract

Background: The cellulose synthase gene superfamily, including the cellulose synthase (*CESA*) and cellulose synthase-like (*CSL*) gene families, is vital for cell wall construction during plant growth, particularly for fiber development of flax, which is an old and important fiber crop.

Results: This study performed a sequencing search of key *CESA* and *CSL* genes from several flax stem parts at different fiber development stages by comparing RNA-Seq. Quantitative RT-PCR was used to validate the expression of these genes. This study revealed that *CESA4* genes (*Lus10008225.g* and *Lus10008226.g*), *CESA6* genes (*Lus10006161.g* and *Lus10041063.g*), *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), *CSLD4* gene (*Lus10026568.g*), *CSLE1* (*Lus10016625.g*) and *CSLG* genes (*Lus10023056.g* and *Lus10023057.g*) were specifically expressed in stem tissue below the snap point where fibers is increased amounts of secondary cell wall deposition. *LusCESA4* genes, *LusCESA8*, genes and *LusCSLD4* gene were specifically expressed in fiber development stage during the fast growth period of flax plants. Based on GO and KEGG analyses, it was found that genes involved in pathways of cellulose microfibril organization, galactosyl transferase activity and galactose metabolism were specifically enriched in the stem tissue of the fiber development stage. Other genes involved in cellulose biosynthesis were also analyzed and discussed.

Conclusion: The results of this study will provide an important foundation for understanding fiber cell wall biogenesis, particularly the roles of *LusCESAs* and *LusCSLs* in the process of fiber development.

Background

Flax (*Linum usitatissimum* L.) is an important bast fiber (phloem fiber) crop that has been domesticated and grown by humankind for many years worldwide. Flax bast fibers are the primary phloem fibers, with the base material being cellulose, which determines its physical and chemical properties and process characteristics [1]. The cellulose content of mature fibers may reach to 70% of their weight [2]. As a model of bast fiber crops, flax is also used for studying both bast fiber differentiation and phloem secondary cell wall development due to its small plant size, short life cycle, self-pollination with numerous seeds, small size of diploid genome (approximately 400Mbp), and can be easily grown at high density in the laboratory [3]. In flax, fiber elongation and cell wall deposition are temporally separated, and secondary cell wall deposition begins immediately after fiber elongation has been completed. The transition point in the stem, where fibers have completed elongation and begin secondary cell wall deposition, is called the snap point [4]. During the fast growth period of flax plants, stem regions above the snap point consist of fibers undergoing elongation, and the region surrounding the snap point contains fibers that are in the transition between elongation and cell wall development; in regions below the snap point, fibers had increasing amounts of secondary cell wall deposition. The fast growth period is critical for flax fiber quality because the maximum length of all bast fibers in the mature plant is determined [1,5-7]. Therefore, it is of great significance to study the expression patterns of genes at different developmental stages of

snap point, especially cellulose synthase-related genes, to better understand the development of fiber cell formation and cell wall thickening.

Cellulose is the most important component of plant cell walls for controlling the directional growth of plant cells [8]. It is biosynthesized at the plasma membrane (PM) by the rosette-structured cellulose synthase (CESA) complex (CSC) [9]. Each transmembrane CSC contains 18 or 36 cellulose synthase protein subunits synthesized by β -1,4-glucan chains using UDP-Glc in the cytosol as a substrate [10]. CESAs are a large protein superfamily that includes nine additional subfamilies named cellulose-like (CSL) defined by sequence similarity to CESAs [11,12]. The genes encoding cellulose synthesis were identified in *A.xylum* for the first time, and CESA genes have been identified in many other species [13-15]. The *Arabidopsis* CESA family members are subdivided into six clusters with orthologous genes from other species, reflecting the functional specialization of distinct CESA isoforms [16,17]. Two functional groups among AtCESAs have been distinguished: *AtCESA1*, *AtCESA3*, and *AtCESA6-like*, which contribute to the synthesis of the primary cell wall (PCW) [18,19] and *AtCESA4*, *AtCESA7*, and *AtCESA8*, which are required during the development of secondary cell wall (SCW) [20,21]. The *Arabidopsis* genome encodes 30 cellulose synthase-like (CSL) genes, which are divided into nine subgroups: CSLA, CSLB, CSLC, CSLD, CSLE, CSLF, CSLG, CSLH and CSLJ, all of which belonging to the glycosyltransferase family 2 (GT2) [22]. CSL genes have been intensively studied due to their implications in the dietary fiber biosynthesis or biomass improvement for bioethanol production [23,24].

High expression levels of CESA genes observed in fast-growing tissues of *L.usitatissimum* indicate that they are particularly important for this fiber crop [25,26]. Currently, 16 flax cellulose synthase genes have been identified based on their homology to orthologous [26-29]. Two genes, *LusCESA7* and *LusCESA9*, were specific to the stems of fiber flax during the “rapid growth” stage [30]. The expression of secondary wall-type *LusCESAs* (*LusCESA4*, *LusCESA7*, and *LusCESA8*) was correlated with the expression of two chitinase-like genes (*LusCTL1* and *LusCTL2*), which were highly enriched in xylem [27]. *LusCESA4* and *LusCESA8* showed higher levels expression in the inner stem tissues than in the outer stem tissues. Both genes were weakly expressed in the flowers, leaves, and roots. *LusCESA3* had significantly higher expression in the outer stem tissues [26]. In our previous study, one CESA4 gene, two CESA8 genes, and two CTL2 genes were found to be enriched in the phloem peel library, and one cellulose synthase-like gene (CSLG3) was detected to be enriched in leaves [31]. Forty-five *LusCESAs* and *LusCSLs* were found to be involved in the flax process of plant development [32]. *LusCSLD3* and *LusCSLG3.2/3.3* genes were highly expressed in samples that were in secondary cell wall development at rapid growth and budding stages [33].

To date, several genome-wide gene expression studies using microarrays and high-throughput sequencing technology have been performed on flax bast fibers, and expressed sequence tags (ESTs) of flax bark tissue have been analyzed [34-38]. However, the cell wall is a highly complex structure, and there must be several CESA and CSL genes involved in the fiber cell wall formation process. Therefore, this study aims to perform expression patterns of CESA and CSL genes related to cell wall formation in the process of fiber elongation and cell wall thickening during the rapid growth period of flax by using RNA-

seq analysis and gene expression profiles. Four sequential time and region points are here investigated. The result of this study will provide a fundamental knowledge for the study of secondary growth and fiber formation in economically important plant.

Materials And Methods

Plant material and collection of stem samples

The elite flax variety Zhongya No.1 was used in this study. Flax seeds were planted in pots with a soil layer of 50cm and grown in a greenhouse at a temperature is between 15°C and 22°C. When plant height was approximately 30cm and 50cm, tissue samples of stem apex, which is above the snap point (SP) and mid-section of stem, which is under the snap point, were collected, and all visible leaves were removed. SP is an indicator of fiber developmental transition from intrusive growth to cell wall thickening, and is easily manually detected from the above stem region by the sharp increase of effort required to break the stem [4]. Approximately 3-5 plants per sample were pooled for RNA extraction. All tissue samples collected were frozen immediately in nitrogen and stored at – 80°C until further processing. Samples above the SP were named ABP, and samples under the snap point were named UNP (Fig.1). Four biologically independent replicates of ABP30 (ABP30_1, ABP30_2, ABP30_3, and ABP30_4), UNP30 (UNP30_1, UNP30_2, UNP30_3, and UNP30_4), ABP50 (ABP50_1, ABP50_2, ABP50_3, and ABP50_4), UNP50 (UNP50_1, UNP50_2, UNP50_3, and UNP50_4) were sequenced on the Illumina HiSeq platform. The other four biologically independent replicates of ABP30 (ABP30_5, ABP30_6, ABP30_7, and ABP30_8), UNP30 (UNP30_5, UNP30_6, UNP30_7, and UNP30_8), ABP50 (ABP50_5, ABP50_6, ABP50_7, and ABP50_8), UNP50 (UNP50_5, UNP50_6, UNP50_7, and UNP50_8) were used for qRT-PCR analysis.

RNA extraction and sequencing

The collected samples were then ground in liquid nitrogen. Total RNA was extracted from the samples using a plant RNA kit (Omega Bio-Tek, USA), and treated with DNase I (OMEGA BIO-TEK, USA) according to the manufacturer's instructions. RNA quality and purity were determined by denaturing 1% agarose gel electrophoresis and a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). The integrity of the extracted RNA was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Sixteen DGE libraries (four for ABP30, four for ABP50, four for UNP30, and four for UNP50) were constructed and sequenced using Illumina technology. The experimental summary process was as follows: a total amount of 3 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Furthermore, mRNA was purified from total RNA using poly T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized

using random hexamer primers and M-MuLV reverse transcriptase (RNase H⁻). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of DNA fragments, NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. To select cDNA fragments of preferentially 250-200 bp in length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Thereafter, 3 µL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min, followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed using the Agilent Bioanalyzer 2100 system. Clustering of the index-coded samples was performed on a cBot cluster. System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform, and 125bp/150bp paired-end reads were generated.

Reads mapping to the reference genome and quantification of gene expression level

All clean reads were aligned to the flax genome database [39]. The annotation of flax genes was performed using Phytozome (phytozome.jgi.doe.gov; downloaded file: *Lusitatisimum_200_v1.0.readme.txt*). The index of the reference genome was built using Bowtie v2.2.3, and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 [40]. TopHat was used as the mapping tool because it can generate a database of splice junctions based on the gene model annotation file and a better mapping result than other non-splice mapping tools [41]. HTSeq v0.6.1 was used to count the read numbers mapped to each gene. Gene expression levels were measured as reads per kilobase of exon model per million mapped reads (RPKM), considering the effect of sequencing depth and gene length for the read count at the same time [42].

Differential expression level analysis

Differential expression analysis of two groups (ABP30 vs. ABP50, ABP30 vs. UNP30, ABP50 vs. UNP50, UNP30 vs. UNP50) was performed using the DESeq R package (1.18.0) [43]. The resulting P-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

Functional annotation of differentially expressed genes (DEGs)

Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was performed using the Goseq R package, in which gene length bias was corrected. GO terms with corrected P-values less than 0.05 were considered significantly enriched by differentially expressed genes. KOBAS was used to test the statistical enrichment of differentially expressed genes in the KEGG pathways [44].

Validation of cellulose synthase genes using qRT-PCR analysis

Ten cellulose synthase genes identified by RNA-Seq were assayed using qRT-PCR. Real-time PCR validation was performed on an optical 96-well plate with a PIKOREAL96 real-time PCR system (Thermo, USA). For each sample, the first-strand cDNAs was reverse transcribed from 1.0 µg total RNA treated with DNase I using reverse transcriptase (Cwbiotech, China) according to the manufacturer's instructions. Each sample contained 2.0 µL of a 1/2 dilution of cDNA in water from the reverse-transcribed reaction mentioned above, 1µL of gene-specific sense primer and 1µL anti primer with the concentration of 10 µM, 15µL of UltraSYBR Mixture (CWbiotech, China) in a final volume of 30µL, and then averaged it out to make each reaction is 10µL and in triplicate for each sample. The thermal cycling condition were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15s, and 60°C for 30s. From each of the four biologically independent cDNA samples, two independent technical replications were performed and averaged for further calculations. Relative expression levels were determined as previously described [45]. The flax CYC gene was selected as the endogenous control [46]. The primer sequences of the DEGs and CYC genes are listed in Table S1.

Statistical analysis

All data are presented as columns with error bars in the figures. All statistical analyses were performed using the statistics software Excel 2010 and SPSS v20.0.

Results

Sequencing-mapping and differentially expressed genes

To characterize the DGE profiles in the ABP and UNP segments of the flax stem, 16 DGE libraries were collected and used for RNA-Seq analysis (Fig.2A). Approximately 47.1–65.0 million raw reads were obtained from these 16 libraries. More than 90% of the reads were mapped to the reference database for the 16 libraries, except UNP30_2. A total of 43,471 genes were detected in all of 16 samples. The RPKM values for each gene in each sample are listed in Table S2. The correlation analysis showed that the correlation between ABP30 and ABP50 was closer than that between ABP30 and UNP30, as well as between ABP30 and UNP50. The correlation between UNP30 and UNP50 was closer than that between ABP30 and UNP30, as well as between ABP30 and UNP50. This indicated that different genes regulate stem growth with different stem regions which above or below the snap point in the same stage. However, the same genes were involved in the growth of the same stem regions at different stem developmental stages. The correlation coefficients of four biologically independent replicates were all above 0.90, except UNP30_1 and UNP30_2, as well as ABP30_1 and ABP30_4, which were 0.899 and 0.829, suggesting that the expression levels of genes in the four replicate libraries were similar and suitable for downstream analysis (Fig.2B).

To identify genes that were differentially expressed in the four tissues of flax, pairs of DGE profiles of the sixteen libraries were compared to analyze gene expression variations. Genes that showed significant differences in expression were identified between the pairwise comparisons of the ABP30 vs. ABP50, ABP30 vs. UNP30, ABP50 vs. UNP50, and UNP30 vs. UNP50 (Fig.2C and Fig.2D). A total of 8639 DEGs

were detected across all four sample types. Among of them, 92% of DEGs were detected in ABP30 vs. UNP30 and UNP30 vs. UNP50. For ABP30 vs. UNP30, there were 2003 up-regulated genes and 1698 down-regulated DEGs. For UNP30 vs. UNP50, there were 2791 up-regulated genes and 1631 down-regulated DEGs. The data suggested that UNP30 had great differences in gene regulation with both ABP30 and UNP50, indicating significant differences between genes that regulate stem regions below the snap point in different stem development stages, including fiber development stage and fiber maturity stages. In the fiber development stage, there are also significant differences between genes that regulate stem regions above the snap point and genes that regulate regions below the snap point. Only 18 up-regulated genes and 12 down-regulated DEGs were detected in ABP30 vs. ABP50, indicating that there are much fewer gene regulating differences than regions below the snap point in different stem development stages. Only 230 up-regulated genes and 256 down-regulated DEGs were detected in ABP50 vs. UNP50, indicating that there are much fewer differences between genes that regulate stem regions above the snap point and genes that regulate regions below the snap point in the fiber mature stage than in fiber development stage. All of detected DEGs with their functional annotations are listed in Table S3.

Most abundant genes in fiber development and maturing stage

For pairwise comparisons of the ABP30 and UNP30, the most abundant transcripts in the stem tissue above the snap point when plant at 30 cm were those of the genes encoding rhamnogalacturonate lyase (*Lus10011565.g*), 1-aminocyclopropane-1-carboxylate synthase 8 (*Lus10002795.g*), fasciclin-like arabinogalactan protein (*Lus10002984.g*, *Lus10036112.g*, and *Lus10036113.g*), leucine-rich repeat extensin-like protein 4 (*Lus10033672.g*), peroxidase (*Lus10020994.g* and *Lus10009932.g*), CASP-like protein 1D1 (*Lus10012808.g*), protein SRG1(*Lus10015252.g*), and MLP-like protein 328 (*Lus10008932.g*). For pairwise comparisons of the UNP30 vs. UNP50, the most abundant transcripts in stem tissue above the snap point when plant at 30 cm (fiber development stage) were those of the genes encoding probable protein kinase At2g41970 (*Lus10000773.g*), RPW8-like protein 3 (*Lus10000836.g*), 1-aminocyclopropane-1-carboxylate oxidase 5 (*Lus10000857.g*), subtilisin-like protease SBT1.6 (*Lus10002397.g*), cytochrome P450 82C4 (*Lus10003148.g*), and MLP-like protein 423 (*Lus10003451.g*). Some genes were highly expressed in the fiber development stage compared to the fiber mature stage but with unknown proteins, such as *Lus10000527.g*, *Lus10000834.g*, *Lus10000835.g*, *Lus10002396.g*, and *Lus10008019.g* (Table 1).

For pairwise comparisons of the ABP50 and UNP50, the most abundant transcripts in stem tissue above the snap point when plants were 50 cm were those of the genes encoding MLP-like protein 328 (*Lus10008930.g*), ethylene-responsive transcription factor ERF022 (*Lus10002953.g*), cytokinin hydroxylase (*Lus10033555.g*), high-affinity nitrate transporter 2.4 (*Lus10026527.g*), cytochrome P450 83B1 (*Lus10034505.g*), subtilisin-like protease SBT1.7 (*Lus10006307.g*), dehydration-responsive element-binding protein 1D (*Lus10027412.g*), and 7-deoxyloganetin glucosyltransferase (*Lus10032218.g*). Two genes, *Lus10015036.g* and *Lus10041692.g* were highly expressed in stem tissue below the snap point with unknown proteins. For pairwise comparisons of the UNP30 vs. UNP50, the most abundant transcripts in stem tissue above the snap point when plants at 50 cm (fiber maturing

stage) were those of the genes encoding 36.4 kDa proline-rich protein (*Lus10010482.g*), transmembrane protein 205 (*Lus10000723.g*), auxin-responsive protein SAUR21 (*Lus10008992.g*), transmembrane protein 205 (*Lus10021473.g*), BTB/POZ and TAZ domain-containing protein 1 (*Lus10033038.g*), anthranilate N-methyltransferase (*Lus10002668.g*), Syntaxin-71 (*Lus10007411.g*) and short-chain dehydrogenase TIC 32, chloroplastic (*Lus10025321.g*). Two genes, *Lus10015276.g* and *Lus10033722.g* were highly expressed in the fiber mature stage compared to the fiber development stage but with unknown proteins (Table 2).

Gene ontology and metabolic pathway enrichment analysis of differentially expressed genes

Using GO analysis, this study classified differentially expressed genes based on enriched GO terms in the biological process, cellular component, and molecular function ontologies. The top 30 significantly enriched categories for the DEGs are listed in Fig. 3. For the stem tissue above the snap point vs. other tissues, cellular components enriched fewer DGEs than the other two ontologies. At the fiber development stage, most of the cellular component genes were enriched above the snap point (ABP30 vs. UNP30). However, at the fiber maturation stage, genes which involved in membrane part, integral component of membrane, intrinsic component of membrane, and intracellular are significantly different between stem tissues above and under the snap point (ABP50 vs. UNP50). For different fiber development stages, most DGEs were enriched in metabolic process, primary metabolic process, catalytic activity, and organic substance metabolic process, and fiber development stage enriched more DEGs than fiber maturing stage for each ontology (UNP30 vs. UNP50). Furthermore, it was found that some cellulose synthesis GO terms are specific in stem tissue of fiber development stage (UNP30), including cellulose microfibril organization (GO:0010215: *Lus10034670.g* and *Lus10017863.g*), galactosyl transferase activity (GO:0008378: *Lus10023955.g*, *Lus10021538.g*, *Lus10039657.g* and *Lus10027185.g*) (Table S4).

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to identify major or specific metabolic pathways and their underlying genes in the fiber development stage (Fig. 4). The term “Biosynthesis of secondary metabolites” was significantly enriched in all of the stem tissue above the snap point vs. other tissues, including over 500 DEGs underlying ABP30 vs. UNP30, UNP30 vs. UNP50, and ABP50 vs. UNP50. In particular, the pathways of “Phosphatidylinositol signaling system,” “Zeatin biosynthesis,” “Glycosaminoglycan degradation,” and “Ubiquitin mediated proteolysis” were significantly enriched in the stem tissue above the snap point. Galactose metabolism pathway is specifically enriched in stem tissue above the snap point in fiber development stage, which include 8 genes (*Lus10003875.g*, *Lus10001822.g*, *Lus10043044.g*, *Lus10002740.g*, *Lus10007370.g*, *Lus10001529.g*, *Lus10034218.g*, *Lus10020788.g*) (Table S5), suggesting genes encoding β -galactosidase participated in the early fiber synthesis.

Differentially expressed genes involved in the cellulose biosynthesis

Cellulose is synthesized by large multi-meric cellulose synthase (CesA) complexes, tracking along cortical microtubules at the plasma membrane (Fig. 5A). Recent studies have identified the Korrigan (KOR), sucrose synthase (SuSy), and COBRA-like proteins are indirectly involved in cellulose biosynthesis.

According to GO function, this study determined the DEGs involved in the pathway of cellulose synthase activity, cellulose biosynthetic process, cellulose metabolic process, cellulose binding, and cellulose microfibril organization. Most of these genes were highly expressed at the fiber development stage (UBP30), comparing with those at the fiber maturing stage (UBP50) or stem tissues above the snap (ABP30 and ABP50), except *Lus10034665.g* and *Lus10020414.g*, which encoding Alpha-1,4-glucan-protein and Phosphoglucosamine mutase (Fig. 5B).

Analysis of CESA and CSL genes in fiber cell wall formation stage

Among the 8639 up-regulated and down-regulated DEGs detected across all four types of samples, 15 genes encoding cellulose synthase proteins or cellulose synthase-like proteins were found. These genes were found in pairwise comparisons of ABP30 vs. UNP30 and UNP30 vs. UNP50. Pairwise comparisons of ABP30 vs. ABP50 and ABP50 vs. UNP50 did not reveal genes related to cellulose synthase. In pairwise comparison of ABP30 vs. UNP30, one probable cellulose synthase A catalytic subunit 3 gene (*Lus10022449.g*) was found to be expressed at significantly higher levels in stem tissue above the snap point than that of below the snap point. In contrast, five cellulose synthase genes, including two *CESA4* genes (*Lus10008225.g* and *Lus10008226.g*), one *CSLD4* gene (*Lus10026568.g*), and two *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), were found to be expressed at significantly higher levels in stem tissue below the snap point than in those above the snap point. In pairwise comparison of UNP30 vs. UNP50, seven cellulose synthase genes, including two *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), one *CESA4* gene (*Lus10008226.g*), one *CSLE1* gene (*Lus10016625.g*), two *CSLG3* genes (*Lus10023056.g* and *Lus10023057.g*), and one *CSLD4* gene (*Lus10026568.g*) were found to be expressed significantly higher in stem tissue with fiber development stage than in the fiber mature stage. In contrast, two *CESA6* genes (*Lus10006161.g* and *Lus10041063.g*) were found to be expressed at significantly higher levels in stem tissue at the fiber mature stage than at the fiber development stage. To confirm the results of expression profiling by RNA-Seq analysis, ten transcripts of cellulose synthase genes were used as targets for quantitative real-time RT-PCR analysis, including two *CESA4* genes (*Lus10008225.g* and *Lus10008226.g*), two *CESA6* genes (*Lus10006161.g* and *Lus10041063.g*), two *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), two *CSLG* genes (*Lus10023056.g* and *Lus10023057.g*), *CSLE1* (*Lus10016625.g*), and *CSLD4* (*Lus10026568.g*). The results showed that the expression patterns of two *CESA4* genes (*Lus10008225.g* and *Lus10008226.g*), two *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), *CSLE1* (*Lus10016625.g*), and *CSLD4* (*Lus10026568.g*) were determined by qRT-PCR fitted in well with that of RNA-seq analysis (Fig. 6A). Furthermore, these six genes were highly expressed in stem tissue at the fiber development stage than in the other three samples, suggesting that these genes were enriched during the fiber cell wall formation stage (Fig. 6B).

Discussion

The CESA genes

Cellulose synthesis is one of the major ongoing processes in flax phloem fibers during the cell wall formation [36]. It is performed by a multi-enzyme complex containing several types of highly homologous and distinct catalytic subunits, such as CESA glycosyltransferases [47]. In our previous study, one CESA4 (*Lus10008226.g*) gene and CESA 8 (*Lus10007296.g* and *Lus10029245.g*) genes were detected to be enriched in the phloem peel library, revealing that CESA4 and CESA8, which belong to secondary wall-type LusCESAs, are important for phloem fiber development [31]. In the present study, six cellulose synthesis machinery genes were detected in pairwise comparisons of analyzed samples, including one probable cellulose synthase A catalytic subunit 3 (PCESA3) (*Lus10022449.g*), two CESA4 genes (*Lus10008225.g* and *Lus10008226.g*), two CESA6 genes (*Lus10006161.g* and *Lus10041063.g*), and two CESA8 genes (*Lus10007296.g* and *Lus10029245.g*). PCESA3 was specifically expressed in stem tissue above the snap point. CESA4 genes (*Lus10008225.g* and *Lus10008226.g*) and CESA8 genes (*Lus10007296.g* and *Lus10029245.g*) were specifically expressed in stem tissue below the snap point when plants were 30cm. CESA6 genes (*Lus10006161.g* and *Lus10041063.g*) were specifically expressed in stem tissue below the snap point when plants were 50cm. Similar to our findings, CESA4 and CESA8 were more enriched in the basal region than in the apical region of the flax stem [35]. Despite the very intensive cellulose synthesis in fibers, Gorshkov et al., (2017) reported that none of the CESA genes were significantly up-regulated in fibers relative to their levels in other samples or present among the most highly expressed genes [36]. In a study by Gorshkova et al., (2018), CESA3 (*Lus10007538*, *Lus10012198*) and CESA6 (*Lus10041063*), which belong to primary wall-type LusCESAs, were moderately up-regulating in intrusively growing fibers above the snap point [37]. Construction of co-expression networks for LusCESAs revealed that both primary and secondary cell wall-related CESAs were involved in the joint co-expression group in fibers depositing tertiary cell walls. The set of secondary wall-type LusCESAs (*LusCESA4*, *LusCESA7*, and *LusCESA8*) is always expressed in cells with thick cell walls [48]. In summary, all of the previous studies and our findings suggest that CESA4 genes (*Lus10008225.g* and *Lus10008226.g*), CESA8 genes (*Lus10007296.g* and *Lus10029245.g*), and CESA6 genes (*Lus10006161.g* and *Lus10041063.g*) crucially participated in the regulation of cell wall deposition stage of fibers and should be further studying using gene-editing strategies.

The CSL genes

At present, 30 CSL genes have been subdivided into nine subfamilies: CSLA to CSLJ [47,49,50]. The CSL genes are likely to be responsible for the biosynthesis of the hemicellulose polymers [51]. Among them, CSLA synthesizes the backbone of mannan or glucomannan [52,53]. CSLC catalyzes the synthesis of xyloglucan, which is a major hemicellulose in the primary walls of many land plants [54]. CSLE is only found in angiosperms, and its function is poorly understood until now [51] (Yin et al., 2014). CSLD might act as a mannan synthases, however, the exact polymer products of CLSD are still under debate [51,55]. CSLF and CSLH, which are found only in grasses, are involved in the synthesis of mixed linkage glucans [56,57]. The functions of other CSLs currently remain unknown [57,58]. Twenty-five LusCSL genes have been identified according to the flax and *Arabidopsis* genomes, including one LusCSLB, 18 LusCSLDs, one LusCSLE, and five LusCSLGs [33]. LusCSLD3 and LusCSLG3.2/3.3 (*Lus10023057.g*) were strongly expressed in the stem sample below the snap point in seedlings, rapid growth, and budding stages.

According to RNA-Seq data, the LusCSLG genes are expressed in the phloem fibers and their expression is practically absent in the xylem [36,59]. In the present study, two CSLG3 genes (*Lus10023056.g* and *Lus10023057.g*) were found to be expressed at significantly higher levels in stem tissue at the fiber development stage than in the fiber mature stage. This result further verified their participation in the flax fiber formation processing. This study hypothesized that CSLG3 encodes a fiber-specific glycosyltransferase involved in cellulose synthases with secondary cell wall-related CESAs, especially CESA4 and CESA8. Furthermore, it was found that LusCSLD4 gene (*Lus10026568.g*) was specifically expressed in stem tissue below the snap point during the fiber development stage, and its expression was significantly higher both than that of stem tissue with mature fiber stage and stem tissue above the snap point. In evolutionary analysis, CSLD and CESA have a closer evolutionary relationship than other family members [32], indicating that it possibly has a similar effect on the synthesis of mixed-linkage glucan. Therefore, it is reasonable to assume that LusCSLD4 (*Lus10026568.g*) plays an important role in the early phloem fiber development stage. Aside from LusCSLD, one CSLE1 gene (*Lus10016625.g*) was also found expressed at significantly higher levels in stem tissue at the fiber development stage than in the fiber mature stage. In a study by Galinousky et al., (2020), LusCSLE1 had the same expression as LusCESA1 and LusCESA6, which encode cellulose synthases responsible for primary cell wall development [33]. It is well known that the primary cell wall from fiber cells should allow the intensive cell wall extension process, which is very important for fiber elongation at the intrusive growth stage [60]. Based on these, the authors can presume that *LusCESE1* participates in the development of the secondary cell wall deposition stage, however, its functions require further research.

Other genes involved in cellulose biosynthesis

Apart from CESA and CSL genes, a number of gene families have been characterized to be involved in cell elongation as well in the different phases of cell wall deposition and thickening in flax by phylogenetic and expression studies, particularly in fiber cells: β -galactosidase (GH35) [61,62]; UDP-glycosyl transferase[63]; rhamnogalacturonan lyases[64]; pectinesterase (PME and PME1) [65]; chitinase-like proteins [27]; tubulin and actin for the cytoskeleton [66,67]. In our previous study, it was found that rhamnogalacturonate lyase (RhiE), COBRA-like protein 4 (COBL4), and endo- β -1,4-glucanase (KOR) may play important roles in the secondary wall deposition [31]. In this study, COBL4 genes (*Lus10017863.g*, *Lus10031972.g*, *Lus10035131.g*, and *Lus10034670.g*) and KOR (*Lus10012162.g*) were moderately up-regulated in stem tissue below the snap point at the fiber development stage (Fig. 5). Gorshkov et al., (2017) found that one COBL4 gene (*Lus10035131.g*) was significantly up-regulated in phloem fibers isolated from stem peels below the snap point [36]. The COBL4 gene encodes a putative glycosyl-phosphatidylinositol-anchored protein that regulates cellulose deposition and oriented cell expansion in the plant cell wall, and has a carbohydrate-binding, which modules cellulose assembly and cellulose microfibril crystallinity [68]. Based on the above research results, this study believes that COBL4 gene plays an important role in the early stage of secondary cell wall deposition and should draw our attention. The gene encoding rhamnogalacturonate lyase (*Lus10004281.g*) was highly expressed in stem tissue below the snap point when the plant was 30 cm. These results are consistent with those of Gorshkov et al., (2017) and Gorshkova et al., (2018), in which rhamnogalacturonan lyase protein family genes were

highly expressed during cellulose deposition rather than intrusively growing fibers, which are mainly above the snap point [36,37]. In addition, some genes are probably involved in β -1,4-galactan and β -(1 $\rightarrow$ 4)-Gal side-chains to the rhamnogalacturonan lyase backbone, such as GALACTAN SYNTHASE 1 (GALS1). A speculative GALS1 gene (*Lus10038387*) was highly expressed in stem tissue below the snap point, especially when the plant was 30 cm, and its expression became weak when the plant was 50 cm (Fig. 5). GALS1 specifically forms β -1,4-galactosyl linkages and can add successive β -1,4-galactosyl residues to the acceptor [69]. In a previous study, *Lus10038387* showed the most evident pattern of specific transcript enrichment in the phloem fiber sample [36]. Our study also showed that *Lus10038387* plays an important role in the early stage of fiber development stage.

Conclusion

In this study, the transcriptome profiles of flax stem regions above SP and under SP at two stem development stages, specifically the rapid growth stage and the maturing stage, were investigated to reveal the expression patterns of *CESA* and *CSL* genes related to cell wall formation during the process of fiber elongation and cell wall thickening during the rapid growth period of flax. The results were verified using qRT-PCR test and showed that *CESA4* (*Lus10008225.g* and *Lus10008226.g*), *CESA8* genes (*Lus10007296.g* and *Lus10029245.g*), *CSLD4* (*Lus10026568.g*), *CSLE1* (*Lus10016625.g*), and *CSLG3* (*Lus10023056.g* and *Lus10023057.g*) were expressed in stem tissue below SP containing fibers with increasing amounts of secondary cell wall deposition. *CESA6* genes (*Lus10006161.g* and *Lus10041063.g*) were specifically expressed in stem tissue below SP when fibers were maturing. *CESA4* genes, *CESA8* genes, and *LusCSLD4* gene were specifically expressed in fiber development stage during the fast growth period of flax plants. Expression of other genes that are involved in cellulose biosynthesis, such as *COBL4* genes (*Lus10017863.g*, *Lus10031972.g*, *Lus10035131.g*, *Lus10034670.g*), *KOR* (*Lus10012162.g*), and *GOLS1* (*Lus10038387*), were also highly and specifically expressed in the stem below the SP at the fiber cell wall thickening stage. This study provides comprehensive information on the *CESA* and *CSL* genes and should aid in the understanding of the molecular basis of agronomically important traits of flax, such as fiber quality and other physiological processes.

Abbreviations

CESA: cellulose Synthase; *CSL*: cellulose synthase-like; qRT-PCR: quantitative real-time PCR; SP: the snap point; PM: plasma membrane; CSC: cellulose synthase complex; ESTs: expressed sequence tags; PCW: primary cell wall; SCW: secondary cell wall; GT2: glycosyltransferase family 2; CTL: chitinase-Like; DGE: digital gene expression; DEGs: differentially expressed genes; RPKM: reads per kilobase of exon model per million mapped reads; COBRA: COBRA-like protein; RhiE: rhamnogalacturonate lyase; KOR: endo- β -1,4-glucanase; GALS1: Galactan Synthase 1.

Declarations

Ethics approval and consent to participate

This study has not directly involved humans or animals. No specific permission was required for use of these materials for experimental purposes. The seedlings were grown in the experimental field of Institute of Bast Fiber Crops, Changsha, China as per standard practices, and samples were harvested at the required time. We comply with the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication

Not applicable.

Availability of data and materials

The datasets for supporting the conclusions of this article are listed in the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

All data generated or analyzed during this study are included in the article with its supplementary material. We have deposited our transcriptome data in Sequence Read Archive (SRA) (<http://www.ncbi.nlm.nih.gov/sra/>), the accession number for our submission is: PRJNA723911.

Authors' contributions

Y.G. performed the experiments and writing-original draft; L. W., J.C., G.P., and Z.W. participated in the sample collection and RNA extraction; Z. L., H. W., Q. X., and C. Q. conducted bioinformatic analysis. S. L. and X. Z. analyzed most of the data; Y. W. provided the materials and financial supporting. All the authors reviewed the draft and approved the final manuscript.

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References

1. Gorshkova TA, Salnikov VV, Pogodina NM, Chemikosova SB, Yablokova EV, Ulanov AV, Ageeva MV, Van Dam J, Lozovaya VV. Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissimum*) stem tissues. *Annals of botany* 2000;85(4):477-486. <https://doi.org/10.1006/anbo.1999.1091>.
2. Titok V, Leontiev V, Shostak L, Khotyleva L. Thermogravimetric analysis of the flax bast fibre bundle. *J. Nat. Fibers* 2006;3(1):35-41. https://doi.org/10.1300/J395v03n01_04.
3. Bennett MD, Leitch IJ. Nuclear DNA amounts in angiosperms. *Annals of botany* 1995;76(2):113-176. <https://doi.org/10.1006/anbo.1995.1085>.
4. Gorshkova TA, Sal'nikov VV, Chemikosova SB, Ageeva MV, Pavlencheva NV, Van Dam JEG. The snap point: a transition point in *Linum usitatissimum* bast fiber development. *Ind. Crop Prod.* 2003;18(3):213-221. [https://doi.org/10.1016/S0926-6690\(03\)00043-8](https://doi.org/10.1016/S0926-6690(03)00043-8).
5. Day A, Ruel K, Neutelings G, Crnier D, David H, Hawkins S, Chabbert B. Lignification in the flax stem: evidence for an unusual lignin in bast fibers. *Planta* 2005;222(2):234-245. <https://doi.org/10.1007/s00425-005-1537-1>.
6. Gorshkova TA, Wyatt SE, Salnikov VV, Gibeaut DM, Ibragimov MR, Lozovaya VV, Carpita NC. Cell-wall polysaccharides of developing flax plants. *Plant physiol.* 1996;110:721-729. <https://doi.org/10.1104/pp.110.3.721>.
7. Mooney C, Stolle-Smits T, Schols H, De Jong E. Analysis of retted and non retted flax fibres by chemical and enzymatic means. *Biotechnol.* 2001;89(2-3):205-216. [https://doi.org/10.1016/S0168-1656\(01\)00299-1](https://doi.org/10.1016/S0168-1656(01)00299-1).
8. Vanholme B, Desmet T, Ronsse F, Rabaey K, Breusegem FV, Mey MD, Soetaert W, Boerjan W. Towards a carbon-negative sustainable bio-based economy. *Front. Plant Sci.* 2013;4(2):174. <https://doi.org/10.3389/fpls.2013.00174>.
9. Morgan JLW, Strumillo J, Zimmer J. Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature* 2013;493(7431):181-192. <https://doi.org/10.1038/nature11744>.
10. Chris S. Cellulose Synthesis in higher plants. *Annu. Rev. Cell. Dev. Bi.* 2006;22(1):53-78. <https://doi.org/10.1146/annurev.cellbio.22.022206.160206>.
11. Doblin MS, Kurek I, Jacob-Wilk D, Delmer DP. Cellulose biosynthesis in plants: from genes to rosettes. *Plant cell physiol.* 2002;43(12):1407-1420. <https://doi.org/10.1093/pcp/pcf164>.

12. Hamann T, Osborne E, Youngs HL, Misson J, Nussaume L, Somerville C. Global expression analysis of *CESA* and *CSL* genes in *Arabidopsis*. *Cellulose* 2004;11(3):279-286. <https://doi.org/10.1023/B:CELL.0000046340.99925.57>.
13. Ross P, Mayer R, Benziman M. Cellulose biosynthesis and function in bacteria. *Microbio. Rev.* 1991; 55(1):35-58. <https://doi.org/10.1128/MR.55.1.35-58.1991>.
14. Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM. Higher plants contain homologs of the bacterial *celA* genes encoding the catalytic subunit of cellulose synthase. *Proc. Natl. Acad. Sci. U. S. A.* 1996;93(22):12637-12642. <https://doi.org/10.1073/pnas.93.22.12637>.
15. Richmond TA, Somerville CR. The cellulose synthase superfamily. *New Phytol.* 2000;124(2):495-498. <https://doi.org/10.1104/pp.124.2.495>.
16. Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR. Interactions among three distinct CesaA proteins essential for cellulose synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 2003;100(3):1450-1455. <https://doi.org/10.1073/pnas.0337628100>.
17. Nairn CJ, Haselkorn T. Three loblolly pine CesaA genes expressed in developing xylem are orthologous to secondary cell wall CesaA genes of angiosperms. *New Phytol.* 2005;166(3):907-915. <https://doi.org/10.1111/j.1469-8137.2005.01372.x>.
18. Desprez T, Juraniec M, Crowell EF, Jouy H, Pochylova Z, Parcy F, Hofte H, Gonneau M, Vernhettes S. Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* 2007;104(39):15572-15577. <https://doi.org/10.1073/pnas.0706569104>.
19. Persson S, Paredez A, Carroll A, Palsdottir H, Doblin M, Poindexter P, Khitrov N, Auer M, Somerville CR. Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Natl. Acad. Sci. U. S. A.* 2007;104(39):15566-15571. <https://doi.org/10.1073/pnas.0706592104>.
20. Hernández-Blanco C, Feng DX, Hu J, Sánchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sánchez-Rodríguez C, Anderson LK, Somerville S, Marco Y, Molina A. Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. *Plant cell* 2007;19(3):890-903. <https://doi.org/10.1105/tpc.106.048058>.
21. Atanassov II, Pittman JK, Turner SR. Elucidating the mechanisms of assembly and subunit interaction of the cellulose synthase complex of *Arabidopsis* secondary cell walls. *Biol. Chem.* 2009;284(6):3833-3841. <https://doi.org/10.1074/jbc.M807456200>.
22. Speicher T, Li P, Wallace I. Phosphoregulation of the plant cellulose synthase complex and cellulose synthase-like proteins. *Plants* 2018;7(3):52. <https://doi.org/10.3390/plants7030052>.
23. Loque D, Scheller HV, Pauly M. Engineering of plant cellwalls for enhanced biofuel production. *Opin. Plant. Biol.* 2015; 25:151-161. <https://doi.org/10.1016/j.pbi.2015.05.018>.
24. Marcotuli I, Colasuonno P, Blanco A, Gadaleta A. Expression analysis of cellulose synthase-like genes in durum wheat. *Scientific report* 2018; 8(1). <https://doi.org/10.1038/s41598-018-34013-6>.

25. Gorshkova TA, Ageeva M, Chemikosova S, Salnikov V. Tissue-specific processes during cell wall formation in flax fiber. *Plant biosys.* 2005;139(1):88-92. <https://doi.org/10.112635005000560> 70.
26. Chantreau M, Chabbert B, Billiard S, Hawkins S. Neutelings G. Functional analyses of cellulose synthase genes in flax (*Linum usitatissimum*) by virus-induced gene silencing. *Plant Biotechnol. J.* 2015;13(9):1312-1324. <https://doi.org/1111/pbi.12350>.
27. Mokshina N, Gorshkova T, Deyholos MK. Chitinase-like (*CTL*) and cellulose synthase (*CESA*) gene expression in gelatinous-type cellulosic walls of flax (*Linum usitatissimum*) bast fibers. *PLoS One* 2014;9 (6):e97949. <https://doi.org/10.3390/fib6020020>.
28. Yurkevich OY, Kirov IV, Bolsheva NL, Rachinskaya OA, Grushetskaya ZE, Zoschuk SA, Samatadze TE, Bogdanova MV, Lemesh VA, Amosova AV, Muravenko OV. Integration of physical, genetic, and cytogenetic mapping data for cellulose synthase (*CesA*) genes in flax (*Linum usitatissimum*). *Front. Plant Sci.* 2017; 8:1467. <https://doi.org/10.3389/fpls.2017.01467>.
29. Galinovsky D, Padvitski T, Bayer G, Pirko Y, Pydiura N, Anisimova N, Nikitinskaya T, Khotyleva L, Yemets A, Kilchevsky A, Blume Y. Expression analysis of cellulose synthase and main cytoskeletal protein genes in flax (*Linum usitatissimum*). *Cell Biol. Int.* 2019;43(9):1065-1071. <https://doi.org/10.1002/cbin.10837>.
30. Galinovskii DV, Anisimova NV, Raiskii AP, Leont'Ev VN, Tito VV, Hotyleva LV. Cellulose synthase genes that control the fiber formation of flax (*Linum usitatissimum*). *Genetika* 2014; 50(1):26-34. <https://doi.org/10.1134/S1022795414010050>.
31. Guo Y, Qiu C, Long S, Chen P, Hao D, Preisner M, Wang H, Wang Y. Digital gene expression profiling of flax (*Linum usitatissimum*) stem peel identifies genes enriched in fiber-bearing phloem tissue. *Gene* 2017;626:32-40. <https://doi.org/10.1016/j.gene.2017.05.002>.
32. Yuan HM, Guo WD, Zhao LJ, Yu Y, Wu JZ, Zhang LG, Cheng LL, Zhao DS, Wu GW, Guan FZ, Bioinformatics and expression analysis of the cellulose synthase supergene family in flax. *Scientia Agricultura Sinica.* 2016;49(23):4656-4668. <https://doi.org/10.3864/j.issn.0578-1752>. (in Chinese).
33. Galinovsky D, Padvitski T, Mokshina N, Gorshkov O, Khotyleva L, Gorshkova T, Kilchevsky A, Expression of cellulose synthase-like genes in two phenotypically distinct flax (*Linum usitatissimum*) subspecies. *Genet. Resour. Crop Ev.* 2020;67:1821-1837. <https://doi.org/10.1007/s10722-020-00943-2>.
34. Roach MJ, Deyholos MK. Microarray analysis of developing flax hypocotyls identifies novel transcripts correlated with specific stages of phloem fibre differentiation. *Annals of Botany* 2008; 102(3):317-330. <https://doi.org/10.1093/aob/mcn110>.
35. Zhang N, Deyholos MK. RNASeq analysis of the shoot apex of flax (*Linum usitatissimum*) to identify phloem fiber specification genes. *Plant Sci.* 2016;7. <https://doi.org/10.3389/fpls.2016.00950>.
36. Gorshkov O, Mokshina N, Gorshkov V, Chemikosova S, Gogolev Y, Gorshkova T. Transcriptome portrait of cellulose-enriched flax fibres at advanced stage of specialization. *Plant. Mol. Biol.* 2017; 93(4-5):431-449. <https://doi.org/10.1007/s11103-016-0571-7>.

37. Gorshkova T, Chernova T, Mokshina N, Gorshkov V, Kozlova L, Gorshkov O. Transcriptome analysis of intrusively growing flax fibers isolated by laser microdissection. Rep.-UK. 2018; 8:14570 <https://doi.org/10.1038/s41598-018-32869-2>.
38. Morello L, Pydiura N, Galinousky D, Blume Y, Breviario D. Flax tubulin and CesA superfamilies represent attractive and challenging targets for a variety of genome- and base-editing applications. Integr. Genomic 2020;20:163-176. <https://doi.org/10.1007/s10142-019-00667-2>.
39. Wang Z, Hobson N, Galindo L, Zhu S, Shi D, McDill J, Yang L, Hawkins S, Neutelings G, Datla R, Lambert G, Galbraith DW, Grassa CJ, Geraldles A, Cronk QC, Cullis C, Dash PK, Kumar PA, Cloutier S, Sharpe AG, Wong GKS, Wang J, Deyholos MK. The genome of flax (*Linum usitatissimum*) assembled de novo from short shotgun sequence reads. Plant J. 2012;72(3):461-473. <https://doi.org/10.1111/j.1365-313X.2012.05093.x>.
40. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat. Methods 2012;9(4): 357-359. <https://doi.org/10.1038/nmeth.1923>.
41. Langmead, B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3):R25. <https://doi.org/10.1186/1471-2229-10-71>.
42. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-seq. Nat. Methods 2008;5:621-628. <https://doi.org/10.1038/nmeth.1226>.
43. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010;26(1):139-140. <https://doi.org/10.1093/bioinformatics/btp616>.
44. Mao XZ, Cai T, Olyarchuk JG, Wei LP. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 2005;21(19):3787-3793. <https://doi.org/10.1093/bioinformatics/bti430>.
45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR. Methods 2001;25(4):402-408. <https://doi.org/10.1006/meth.2001.1262>.
46. Huis R, Hawkins S, Neutelings G. Selection of reference genes for quantitative gene expression normalization in flax (*Linum usitatissimum*). BMC Plant Biology 2010;10(1):71. <https://doi.org/10.1186/1471-2229-10-71>.
47. Richmond TA, Somerville CR. Integrative approaches to determining Csl function. Plant Mol. Biol. 2001;47(1):131-143. <https://doi.org/10.1023/A:1010627314782>.
48. Mokshina N, Gorshkov O, Ibragimova N, Chernova T, Gorshkova T. Cellulosic fibres of flax recruit both primary and secondary cell wall cellulose synthases during deposition of thick tertiary cell walls and in the course of graviresponse. plant biol. 2017;44(8):820-831. <https://doi.org/10.1071/FP17105>.
49. Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredes A, Persson S, Raab T, Vorwerk S, Youngs H. Toward a systems approach to understanding plant cell walls. Science 2004;306(5705):2206-2211. <https://doi.org/10.1126/science.1102765>.

50. Farrokhi N, Burton RA, Brownfield L, Hrmova M, Wilson SM, Bacic A, Fincher GB. Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes. *Plant Biotechnol. J.* 2006;4(2):145-167. <https://doi.org/10.1111/j.1467-7652.2005.00169.x>
51. Yin Y, Johns MA, Cao H, Rupani M. A survey of plant and algal genomes and transcriptomes reveals new insights into the evolution and function of the cellulose synthase superfamily. *BMC Genomics* 2014;15:260. <http://dx.doi.org/10.1186/1471-2164-15-260>.
52. Liepman AH, Wilkerson CG, Keegstra K, Kende HJ. Expression of cellulose synthase-like (*Cs*) genes in insect cells reveals that *Cs*LA family members encode mannan synthases. *Proc. Natl. Acad. Sci. U. S. A.* 2005;102(6):2221-2226. <https://doi.org/10.1073/pnas.0409179102>.
53. Goubet F, Barton CJ, Mortimer JC, Yu X, Zhang Z, Miles GP, Richens J, Liepman AH, Seffen K, Dupree P. Cell wall glucomannan in *Arabidopsis* is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. *Plant J.* 2009;60(3):527-538. <https://doi.org/10.1111/j.1365-313X.2009.03977.x>.
54. Cocuron JC, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Raikhel N, Wilkerson CG. A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104(20): 8550-8555. <https://doi.org/10.1073/pnas.0703133104>.
55. Verhertbruggen Y, Yin L, Oikawa A, Scheller HV. Mannan synthase activity in the CSLD family. *Plant Signal. Behav.* 2011;6(10):1620-1623. <https://doi.org/10.4161/psb.6.10.17989>.
56. Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB. Cellulose synthase-like *Cs/F* genes mediate the synthesis of cell wall (1,3;1,4)- β -D-Glucans. *Science* 2006; 11(1940):1940-1942. <https://doi.org/10.1126/science.1122975>.
57. Doblin MS, Pettolino FA, Wilson SM, Campbell R, Burton RA, Fincher GB, Newbigin E, Bacic A. A barley *cellulose synthase-like CSLH* gene mediates (1,3;1,4)-beta-D-glucan synthesis in transgenic *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 2009;106(14):5996-6001. <https://doi.org/10.1073/pnas.0902019106>.
58. Suzuki S, Li L, Sun ., Chiang VL. The cellulose synthase gene superfamily and biochemical functions of xylem-specific cellulose synthase-like genes in *Populus trichocarpa*. *Plant Physiol.* 2006;142(3):1233-1245. <https://doi.org/10.1104/pp.106.086678>.
59. Galinousky D, Padvitski T. Analysis of public RNA-seq data in studies of flax fiber biogenesis. *The Eurobiotech. Journal* 2017;1(2):177-179. <https://doi.org/24190/ISSN2564-615X/2017/02.10>
60. Mokshina N, Chernova T, Galinousky D, Gorshkov O, Gorshkova T. Key stages of fiber development as determinants of bast fiber yield and quality. *Fibers* 2018;6(2):20. <https://doi.org/10.3390/fib6020020>.
61. Roach MJ, Mokshina NY, Badhan A, Snegireva AV, Hobson N, Deyholos MK, Gorshkova TA. Development of cellulosic secondary walls in flax fibers requires β -galactosidase. *Plant Physiol.* 2011;156(3):1351-1363. <https://doi.org/10.1104/pp.111.172676>.
62. Hobson N, Deyholos MK. Genomic and expression analysis of the flax (*Linum usitatissimum*) family of glycosyl hydrolase 35 genes. *BMC Genomics* 2013;14(1):344. <https://doi.org/10.1186/1471->

2164-14-344.

63. Barvkar VT, Pardeshi VC, Kale SM, Kadoo NY, Gupta VS. Phylogenomic analysis of UDP glycosyltransferase 1 multigene family in *Linum usitatissimum* identified genes with varied expression patterns. BMC Genomics 2012;13(1):175. <https://doi.org/10.1186/1471-2164-13-175>.
64. Hotte NSC, Deyholos MK. A flax proteome: identification of proteins enriched in bast fibres. BMC Plant Biol 2008;8(1):52. <https://doi.org/10.1186/1471-2229-8-52>.
65. Pinzón-Latorre D, Deyholos MK. Characterization and transcript profiling of the pectin methylesterase (PME) and pectin methylesterase inhibitor (PMEI) gene families in flax (*Linum usitatissimum*). BMC Genomics 2013;14(1):742. <https://doi.org/10.1186/1471-2164-14-742>.
66. Gavazzi F, Pigna G, Braglia L, Gianì S, Breviario D, Morello L. Evolutionary characterization and transcript profiling of β -tubulin genes in flax (*Linum usitatissimum*) during plant development. BMC Plant Biol. 2017;17(1):237. <https://doi.org/10.1186/s12870-017-1186-0>.
67. Pydiura N, Pirko Y, Galinowsky D, Postovoirova A, Yemets A, Kilchevsky A, Blume Y. Genome-wide identification, phylogenetic classification, and exon-intron structure characterisation of the tubulin and actin genes in flax (*Linum usitatissimum*). Cell Biol Int 2018;43(5). <https://doi.org/10.1002/cbin.11001>.
68. Liu L, Shang-Guan K, Zhang B, Liu X, Yan M, Zhang L, Shi Y, Zhang M, Qian Q, Li J, Zhou Y. Brittle Culm1, a COBRA-like protein, functions in cellulose assembly through binding cellulose microfibrils. Plos. Genet. 2013;9(8):e1003704. <https://doi.org/10.1371/journal.pgen.1003704>.
69. Liwanag AJM, Ebert B, Verherbruggen Y, Rennie EA, Rautengarten C, Oikawa A, Andersen MC F, Clausen MH, Scheller HV. Pectin biosynthesis: *GALS1* in *Arabidopsis thaliana* is a β -1,4-galactan β -1,4-galactosyltransferase. Plant Cell 2012;24(12):5024-5036. <https://doi.org/10.1105/tpc.112.106625>.

Tables

Table 1 Top twenty of down-regulated and up-regulated genes in pairwise comparisons of ABP30 vs. UNP30 and UNP 30 vs. UNP 50, respectively.

Gene ID	log2Fold	Gene	Functional annotation
<i>ABP 30 vs UNP 30 (down-regulated)</i>			
Lus10004281.g	-14.902	RHIE	Rhamnogalacturonate lyase
Lus10011565.g	-11.701	ACS8	1-aminocyclopropane-1-carboxylate synthase 8
Lus10002984.g	-11.696	FLA11	Fasciclin-like arabinogalactan protein 11
Lus10033672.g	-11.132	LRX4	Leucine-rich repeat extensin-like protein 4
Lus10020994.g	-10.567	PER10	Peroxidase 10
Lus10012808.g	-10.556	RCOM_0679870	CASP-like protein 1D1
Lus10015252.g	-10.363	SRG1	Protein SRG1
Lus10008932.g	-10.278	MLP328	MLP-like protein 328
Lus10036112.g	-10.063	FLA12	Fasciclin-like arabinogalactan protein 12
Lus10036113.g	-9.1933	FLA12	Fasciclin-like arabinogalactan protein 12
Lus10016080.g	-9.0561	At3g07570	Cytochrome b561 and DOMON domain-containing protein At3g07570
Lus10031326.g	-8.9001	MYB44	Transcription factor MYB44
Lus10035585.g	-8.8041	At5g03810	GDSL esterase/lipase At5g03810
Lus10029145.g	-8.7309	GOLS1	Galactinol synthase 1
Lus10022577.g	-8.6874	PIP2-1	Aquaporin PIP2-1 OS=Zea mays GN=PIP2-1 PE=1 SV=2//1.33266e-19
Lus10026200.g	-8.6063	NAC021	NAC domain-containing protein 21/22
Lus10005818.g	-8.597	ATL76	E3 ubiquitin-protein ligase ATL76
Lus10028638.g	-8.5858	MYB44	Transcription factor MYB44
Lus10009932.g	-8.4871	GSVIVT00023967001	Peroxidase 4
Lus10012663.g	-8.4764	NPF5.7	Protein NRT1/ PTR FAMILY 5.7
<i>UNP 30 vs UNP 50 (up-regulated)</i>			
Lus10000527.g	Inf		Unknown
Lus10000773.g	Inf	At2g41970	Probable protein kinase At2g41970
Lus10000834.g	Inf		Unknown
Lus10000835.g	Inf		Unknown
Lus10000836.g	Inf	HR3	RPW8-like protein 3

Lus10000857.g	Inf	At1g77330	1-aminocyclopropane-1-carboxylate oxidase 5
Lus10002396.g	Inf		Unknown
Lus10002397.g	Inf	SBT1.6	Subtilisin-like protease SBT1.6
Lus10003148.g	Inf	CYP82C4	Cytochrome P450 82C4
Lus10003451.g	Inf	MLP423	MLP-like protein 423
Lus10004046.g	Inf	At4g08850	Probable LRR receptor-like serine/threonine-protein kinase At4g08850
Lus10004452.g	Inf	HD3A	Protein HEADING DATE 3A
Lus10005104.g	Inf	ATL40	RING-H2 finger protein ATL40
Lus10005178.g	Inf	FMO1	Probable flavin-containing monooxygenase 1
Lus10006692.g	Inf	ROMT	Trans-resveratrol di-O-methyltransferase
Lus10007073.g	Inf		Agglutinin-2
Lus10007890.g	Inf		Miraculin
Lus10007892.g	Inf		Miraculin
Lus10008019.g	Inf		Unknown
Lus10008083.g	Inf	BOR4	Boron transporter 4

“Inf” is abbreviation of infinity.

Table 2 Top twenty of down-regulated genes in pairwise comparisons of ABP50 vs. UNP50 and UNP 30 vs. UNP 50.

Gene ID	log2Fold	Gene	Functional annotation
<i>ABP50 vs UNP 50 (down-regulated)</i>			
Lus10008930.g	-7.9134	MLP328	MLP-like protein 328
Lus10002953.g	-5.8702	ERF022	Ethylene-responsive transcription factor ERF022
Lus10033555.g	-5.3647	CYP735A1	Cytokinin hydroxylase
Lus10026527.g	-5.2991	NRT2.4	High affinity nitrate transporter 2.4
Lus10034505.g	-5.2718	CYP83B1	Cytochrome P450 83B1
Lus10006307.g	-4.9387	SBT1.7	Subtilisin-like protease SBT1.7
Lus10027412.g	-4.8852	DREB1D	Dehydration-responsive element-binding protein 1D
Lus10011872.g	-4.8673	SDI1	Protein SULFUR DEFICIENCY-INDUCED 1
Lus10015036.g	-4.7284		Unknown
Lus10032218.g	-4.708	UGT85A24	7-deoxyloganetin glucosyltransferase
Lus10006668.g	-4.5712	yoaA	Uncharacterized N-acetyltransferase YoaA
Lus10030554.g	-4.5654	At3g19850	BTB/POZ domain-containing protein At3g19850
Lus10021473.g	-4.3737	Tmem205	Transmembrane protein 205
Lus10004180.g	-4.3724	FAD2	Omega-6 fatty acid desaturase, endoplasmic reticulum
Lus10029838.g	-4.2832	LSH10	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 10
Lus10020700.g	-4.1491	LSH10	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 10
Lus10004181.g	-3.9629	FAD2	Omega-6 fatty acid desaturase, endoplasmic reticulum
Lus10015206.g	-3.961	PPC6-7	Probable protein phosphatase 2C 73
Lus10041692.g	-3.8414		Unknown
Lus10026948.g	-3.7975	HKT1	Sodium transporter HKT1
<i>UNP30 vs UNP 50 (down-regulated)</i>			
Lus10010482.g	-9.9803	TPRP-F1	36.4 kDa proline-rich protein
Lus10000723.g	-9.1419	TMEM205	Transmembrane protein 205
Lus10015276.g	-7.1798		Unknown
Lus10008992.g	-7.0571	SAUR21	Auxin-responsive protein SAUR21
Lus10033722.g	-6.8499		Unknown
Lus10021473.g	-6.8354	Tmem205	Transmembrane protein 205

Lus10033038.g	-6.7895	BT1	BTB/POZ and TAZ domain-containing protein 1
Lus10002668.g	-6.7359		Anthranilate N-methyltransferase
Lus10007411.g	-6.7104	SYP71	Syntaxin-71
Lus10025321.g	-6.4556	TIC32	Short-chain dehydrogenase TIC 32, chloroplastic
Lus10010758.g	-6.4018	CYP82A1	Cytochrome P450 82A1 (Fragment)
Lus10035402.g	-6.3424	–	Nitrate reductase [NADH]
Lus10013956.g	-6.2354	At5g45950	GDSL esterase/lipase At5g45950
Lus10023515.g	-6.0237	PIP2-5	Probable aquaporin PIP2-5
Lus10034505.g	-5.9872	CYP83B1	Cytochrome P450 83B1
Lus10000868.g	-5.9356	CPP1	Protein CHAPERONE-LIKE PROTEIN OF POR1, chloroplastic
Lus10004886.g	-5.897	CET4	CEN-like protein 4
Lus10029838.g	-5.87	LSH10	Protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 10
Lus10041484.g	-5.7543	alx-1	Apoptosis-linked gene 2-interacting protein X 1
Lus10032218.g	-5.631	UGT85A24	7-deoxyloganetin glucosyltransferase

Figures

Figure 1

Scheme of sample collection. Segments of flax stem top region (ABP) and middle region (UNP) were isolated when plants were about 30cm and 50cm, respectively. SP: Snap point. Bar=1cm.

Figure 2

RNA-Seq analysis of sixteen stem samples and different expression genes across four types of stem samples. (A) Summary of RNA-Seq mapped to reference genes. (B) The correlation of RPKM values of all genes between the four replicates of ABP30, ABP50, UNP30, UNP50. (C) Number of up-regulated and down-regulated genes in the case of pairwise analysis of ABP30, ABP50, UNP30, UNP50 samples of flax stem. (D) Venn diagram showing distribution and number of specific, common and uniquely expressed genes in the case of a pairwise analysis of ABP30 vs. ABP50 (blue ellipse), ABP30 vs. UNP30 (green ellipse), ABP50 vs. UNP50 (yellow ellipse), UNP30 vs. UNP50 (purple ellipse).

Figure 3

Gene classification based on Gene Ontology (GO) for differentially expressed genes (DEGs) in four types of stem samples.

Figure 4

Kyoto encyclopedia of genes and genomes database (KEGG) functional annotations of differentially expressed genes (DEGs) for four types of stem samples.

Figure 5

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Cellulose biosynthesis and expression profiles of genes involved in cellulose biosynthesis. (A) Plant's cellulose biosynthesis: Cesa complexes (CSC) use Uridine diphosphate-glucose (UDP-Glc) as the activated sugar donor for the β -1,4 glucan chain polymerization. UDP-Glc synthesized by sucrose synthase. Plasma membrane-bound forms of sucrose synthase are implicated in carbon supply for cellulose production. Korrigan cellulase (Kor) has been involved in monitoring of cellulose synthesis. Microtubules (MT) play role to regulate Cesa proteins trafficking (Amor et al., 1995, Doblin et al., 2002, Endler and Persson, 2011). (B) Expression profiles of genes involved in cellulose biosynthesis.

Figure 6

Comparison of CESA and CSL genes expression values obtained by qRT-PCR and RNA-Seq analysis. (A) Quantitative RT-PCR validation of 10 differentially expressed CESA and CSL genes among ABP30, ABP50,

UNP30 and UNP50 revealed by digital gene expression (DGE) libraries of *Linum usitatissimum* L.. The values for the samples are presented as the fold change in gene expression, normalized to the CYC gene. RPKM: Gene expression levels were measured as reads per kilobase of exon model per million mapped reads. CESA: Cellulose synthase A; CSLG, CSLD and CSLE: Cellulose synthase-like protein. Vertical bars represent the mean $\pm$ SD (n=4). (B) Overall and normalized form of expression between qRT-PCR and FPKM for differentially expressed CESA and CSL genes among ABP30, ABP50, UNP30 and UNP50.

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