Chitinase-Like (CTL) and Cellulose Synthase (CESA) Gene Expression in Gelatinous-Type Cellulosic Walls of Flax (Linum usitatissimum L.) Bast Fibers

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Abstract

Plant chitinases (EC 3.2.1.14) and chitinase-like (CTL) proteins have diverse functions including cell wall biosynthesis and disease resistance. We analyzed the expression of 34 chitinase and chitinase-like genes of flax (collectively referred to as LusCTLs), belonging to glycoside hydrolyase family 19 (GH19). Analysis of the transcript expression patterns of LusCTLs in the stem and other tissues identified three transcripts (LusCTL19, LusCTL20, LusCTL21) that were highly enriched in developing bast fibers, which form cellulose-rich gelatinous-type cell walls. The same three genes had low relative expression in tissues with primary cell walls and in xylem, which forms a xylan type of secondary cell wall. Phylogenetic analysis of the LusCTLs identified a flax-specific sub-group that was not represented in any of other genomes queried. To provide further context for the gene expression analysis, we also conducted phylogenetic and expression analysis of the cellulose synthase (CESA) family genes of flax, and found that expression of secondary wall-type LusCESAs (LusCESA4, LusCESA7 and LusCESA8) was correlated with the expression of two LusCTLs (LusCTL1, LusCTL2) that were the most highly enriched in xylem. The expression of LusCTL19, LusCTL20, and LusCTL21 was not correlated with that of any CESA subgroup. These results defined a distinct type of CTLs that may have novel functions specific to the development of the gelatinous (G-type) cellulosic walls.

Introduction

Flax (Linum usitatissimum L.) phloem fibers are a valuable industrial feedstock and are also a convenient model system for studying secondary cell wall formation. The mechanical properties of bast fibers are largely dependent on the composition of their secondary walls. Bast fibers have gelatinous-type walls, which are rich in cellulose (up to 90%) and lack detectable xylan and lignin. Gelatinous fibers are widespread in various land plant taxa, but have been studied primarily in angiosperms. Depending on the species, either phloem or xylem (of either primary or secondary origin) can produce gelatinous fibers in various organs including stems, roots, tendrils, vines, and peduncles [1,2]. The mechanisms of gelatinous cell wall development in these fibers remain largely unclear. However, some genes implicated in gelatinous cell wall development have been identified. The list includes fasciclin-like arabinogalactan proteins (FLAs) [3-6], β-galactosidases [7,8], and lipid transfer proteins [6]. A role for β-galactosidases in G-type wall development has been demonstrated functionally [8].

Transcripts of genes encoding chitinase-like proteins are reportedly enriched in fibers, particularly during the cell wall thickening stage of flax phloem cellulosic fiber development [6]. Expression of GCTs during primary or secondary cell wall deposition has also been reported in species other than flax [9,10]. Plant chitinase-like proteins have been identified in a wide range of organisms and tissues, including the apoplast and vacuole [11].

Chitinase-like proteins belong to a large gene family that includes genuine chitinases (i.e. proteins with proven chitinase activity) and other homologous proteins, which may not have chitinase activity [12-15]. Here, we refer to both genuine chitinases and their homologs collectively as chitinase-like proteins (CTLs).

Chitinases catalyze cleavage of β-1,4-glycoside bonds of chitin and are organized in five classes (Classes I–V), which can be distinguished on the basis of sequence similarity [11,16,17]. Classes I, II, and IV belong to glycoside hydrolase family 19 (GH19), found primarily in plants, whereas Classes III and V belong to glycoside hydrolase family 18 (GH18) present in various types of organisms [18–20]. The Class I chitinases are found in both monocots and dicots, while classes II and IV are found mainly in dicots [21]. Class I and IV chitinases contain a highly-conserved cysteine-rich domain – the chitin binding domain (CBD) – at the N-terminal region [21,22], but there are two characteristic deletions in the main catalytic domain in Class IV chitinases [21]. Because chitin is the major component of fungal cell walls, chitinases are classic pathogenesis-related proteins involved in non-host-specific defense [23,24].

Plants also contain chitinase-like proteins that are not induced by pathogens or stresses. In many cases, these chitinase-like

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proteins have been shown to lack detectable chitinase activity. Chitinase-like proteins may play an important role during normal plant growth and development [13,15,25]. For example, AtCTL1 is constitutively expressed in many organs of Arabidopsis. Mutations of AtCTL1 lead to ectopic deposition of lignin in the secondary cell wall, reduction of root and hypocotyl lengths, and increased numbers of root hairs [15]. It was suggested that this gene could be involved in root expansion, cellulose biosynthesis, and responses to several environmental stimuli [13,26,27]. In particular, co-expression of some CTLs with secondary cell wall cellulose synthases (CESAs) was reported [28]. It has been suggested that these chitinase-like proteins could take part in cellulose biosynthesis and play a key role in establishing interactions between cellulose microfibrils and hemicelluloses [14].

The xylan-type secondary wall is the most common secondary wall in land plants and is characteristically rich in cellulose, xylan, and lignin [2]. Compared to typical xylan-type secondary walls, gelatinous layers are enriched in cellulose, have a higher degree of cellulose crystallinity, larger crystallites, and a distinctive set of matrix polysaccharides (see [2] and references therein). Presumably, cellulose synthase genes have a significant role in gelatinous cell wall formation, but the expression patterns of the complete flax CES family has not been described to date. It is known that at least three isoforms of CESAs comprise the cellulose synthase rosette: CESA1, CESA3, and CESA6 are required for cellulose biosynthesis in primary cell walls [29], whereas CESA4, CESA7, and CESA8 are required for cellulose biosynthesis during secondary wall deposition [30].

Flax is a useful model for comparative studies of cell wall development: different parts of the flax stem form a primary cell wall, xylan type secondary cell wall, or gelatinous cell wall; these stem parts may be separated and analyzed by diverse approaches, including functional genomics. Furthermore, the publication of a flax whole genome assembly [31] facilitates a thorough study of key gene families.

In the present study, we measured expression of all predicted LusCTL genes of the GH19 family in various tissues including those that produce gelatinous-type and xylan-type cell walls. We also described the LusCESA gene family and measured expression of its transcripts in comparison to LusCTLs. Phylogenetic analysis of LusCTL and LusCESA genes identified distinct groups of LusCTL genes that were expressed preferentially at specific stages of bast fiber gelatinous cell wall development.

### Materials and Methods

#### Plant Growth

Flax (Linum usitatissimum L.) var. Mogilevsky plants were grown in pots in a growth chamber at 22°C, with a light intensity of approximately 200 µE on a 16 h light/8 h dark cycle. Plants were harvested at the period of rapid growth (4 weeks after sowing). Plant growth was monitored throughout the experiment, and leaf, stem, and root samples were collected for RNA extraction. The segments of the flax stem (apical part “Apex”, top region TOP, middle region MID, Peel containing phloem fibers, Xylem and isolated Fibers) were taken for RNA extraction. A detailed description of each sample is presented in Table 1.
Table 2. Predicted chitinase-like sequences in *L. usitatissimum*.

| Locus I.D.   | Label | Length, aa | MW, kDa | pI   | Domains* | SP** | Group |
|--------------|-------|------------|---------|------|----------|------|-------|
| Lus10016872  | LusCTL1 | 209        | 22.9    | 6.2  | –        | –    | A     |
| Lus10037737  | LusCTL2 | 330        | 35.9    | 6.7  | –        | +    | A     |
| Lus10037428  | LusCTL3 | 327        | 36.2    | 7.0  | –        | +    | A     |
| Lus10037430  | LusCTL4 | 325        | 36.0    | 6.7  | –        | +    | A     |
| Lus10041278  | LusCTL5 | 325        | 35.9    | 6.7  | –        | +    | A     |
| Lus10041282  | LusCTL6 | 118        | 13.2    | 5.2  | –        | –    | A     |
| Lus10041829  | LusCTL7 | 131        | 14.5    | 7.7  | –        | –    | B     |
| Lus10041830  | LusCTL8 | 320        | 34.4    | 6.9  | CBD     | +    | B     |
| Lus10028378  | LusCTL9 | 131        | 13.8    | 6.0  | –        | –    | B     |
| Lus10028377  | LusCTL10 | 328       | 35.1    | 7.4  | CBD     | +    | B     |
| Lus10041831  | LusCTL11 | 125       | 13.6    | 9.0  | –        | –    | B     |
| Lus10000193  | LusCTL12 | 125       | 13.5    | 8.6  | –        | –    | B     |
| Lus10038026  | LusCTL13 | 274       | 30.2    | 8.8  | –        | +    | B     |
| Lus10009968  | LusCTL14 | 274       | 30.2    | 8.9  | –        | +    | B     |
| Lus10000453  | LusCTL15 | 264       | 28.7    | 4.4  | CBD     | +    | C     |
| Lus1003230   | LusCTL16 | 235       | 26.4    | 8.4  | –        | +    | C     |
| Lus10024367  | LusCTL17 | 223       | 24.6    | 8.5  | –        | +    | C     |
| Lus10010863  | LusCTL18 | 232       | 25.6    | 8.6  | –        | +    | C     |
| Lus10010864  | LusCTL19 | 230       | 25.8    | 8.6  | –        | +    | C     |
| Lus10010866  | LusCTL20 | 224       | 24.8    | 5.1  | –        | +    | C     |
| Lus10024366  | LusCTL21 | 225       | 24.8    | 5.1  | –        | +    | C     |
| Lus10035618  | LusCTL22 | 232       | 25.5    | 8.9  | –        | +    | C     |
| Lus10035620  | LusCTL23 | 389       | 43.4    | 9.0  | –        | +    | C     |
| Lus1003231   | LusCTL24 | 226       | 24.8    | 9.2  | –        | +    | C     |
| Lus10035621  | LusCTL25 | 414       | 44.7    | 7.1  | –        | +    | C     |
| Lus10024369  | LusCTL26 | 226       | 25.1    | 8.7  | –        | +    | C     |
| Lus10035624  | LusCTL27 | 318       | 35.7    | 5.7  | –        | +    | C     |
| Lus10003227  | LusCTL28 | 317       | 35.6    | 5.7  | –        | +    | C     |
| Lus10000217  | LusCTL29 | 193       | 21.6    | 4.4  | –        | +    | C     |
| Lus10035625  | LusCTL30 | 304       | 33.7    | 6.1  | –        | +    | C     |
| Lus10003226  | LusCTL31 | 326       | 36.2    | 4.9  | –        | +    | C     |
| Lus10024368  | LusCTL32 | 229       | 25.0    | 9.9  | –        | +    | C     |
| Lus10010861  | LusCTL33 | 229       | 24.9    | 9.9  | –        | +    | C     |
| Lus10010862  | LusCTL34 | 229       | 25.0    | 9.9  | –        | +    | C     |
| Lus10003587  | LusCTL35 | 223       | 24.1    | 9.5  | –        | +    | C     |
material was sampled with respect to the location of the snap point, which is a mechanically defined stem position in which fibers undergo transition from elongation to secondary cell wall formation [32]. The following seven samples were collected (Table 1, Figure 1): 1. “Apex” – the apical part of stem (1 cm of length). 2. “TOP” – the following “apex” segment of stem above the snap point with phloem fibers in the process of elongation. 3. “MID” – the stem segment (5 cm of length) below the snap point which contained fibers at early stages of secondary cell wall thickening. 10 cm of the stem downwards from “MID” was divided into Peel (4), which contained epidermis, parenchyma cells, phloem fiber bundles and sieve elements and Xylem (5), which contained parenchyma cells, xylem vessels and xylem fibers. 6. “Fibers” – i.e. isolated phloem fibers were obtained by washing Peels in 80% ethanol in a mortar several times and gently pressing the fiber-bearing tissues with a pestle to release the fibers. 7. Roots. The number of biological replicates was three, with five plants in each replicate.

Sequence Alignment and Phylogenetic Analysis
Predicted amino acid and nucleotide sequences of CTLs (Pfam domain: PF00182) and CESAs (PF03552) were obtained from the Phytozome database v.9.0 (Linum usitatissimum, Populus trichocarpa, Arabidopsis thaliana). CESAs of poplar (PtCESAs) were renamed according to Kumar et al. [33]. A list of various well-characterized CTLs from different plant species was obtained from previously published works [10,21]. Sequences were aligned using MUSCLE with default parameters, and a phylogenetic tree was constructed using MEGA5 based on the Maximum Likelihood and Neighbor-Joining methods [34], bootstrapping 1000 replicates [35], model WAG+G or JTT+G. Signal peptides for protein sequences were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/), molecular weights, isoelectric points of the proteins were analyzed by ProtParam (http://web.expasy.org/protparam/).

Reverse Transcription Quantitative Real Time PCR
Total RNA from all plant samples was isolated using a Trizol-extraction method combined with an RNaseasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA quality was evaluated by electrophoresis using a BioAnalyzer (Agilent), and no degradation of RNA was evident. Residual DNA was eliminated by treatment with DNase I using the DNA-free kit (Ambion). Gene specific primers for CTL and CesA genes were designed using Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/shop/CategoryDisplay? catalogId=10001&tab=identifier=Universal+Probe+Library) (File S1). One microgram of total RNA was reverse-transcribed using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNAs were diluted 1:32 with nuclease free water. Real-time PCR was performed in a 7900 HT Fast real-time PCR system (Applied Biosystems, USA). Each 10 µL real-time PCR cocktail contained 2.5 µL of 0.4 µM concentrations of both forward and reverse gene-specific primers, and 2.5 µL of cDNA, 5 µL of 2×Dynamite qPCR mastermix (Molecular Biology Service Unit - University of Alberta) which included SYBR green (Molecular Probes) and Platinum Taq (Invitrogen). The thermal cycling conditions were 95°C for 5 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. A 60–95°C melting curve was performed to confirm the specificity of the products. Threshold cycles (CT) were determined using 7900 Fast Software. C_{T1} values were normalized using eukaryotic translation initiation factors 1A, 5A (LusETIF1, LusETIF5A) and glyceraldehyde 3-phosphate dehydrogenase (LusGAPDH) gene from flax (File S1) [36]. From each of three biologically independent cDNA samples, two independent technical replications were performed and averaged
for further calculations. ΔΔCT values were generated using the apex sample as a reference. Relative transcript abundance calculations were performed using comparative CT (ΔCT) method as previously described [37] for flax tissues (TOP/Apex, MID/Apex, Peel/Apex, Xylem/Apex, Fiber/Apex, Root/Apex). Heat maps of expression levels of some genes were then created with MeV v4.8 (Multi Experiment Viewer, http://www.tm4.org/mev) using the means of ΔCT.

Results

LusCTL Phylogenetic Characterization

We searched within the flax genome assembly (version 1.0) for predicted genes with homology to Pfam domain PF00182, which is characteristic of chitinases of the glycosyl hydrolase family 19 (GH 19) family [31,38]. This search identified 37 predicted chitinase or chitinase-like genes (referred to here collectively as LusCTLs) (Table 2). However, only three of the predicted proteins (LusCTL8, LusCTL10, and LusCTL15) contained a conserved chitin-binding domain (CBD), suggesting that not all of the LusCTLs use chitinase as a substrate. The mean predicted protein size of the 37 LusCTLs was 246.5 aa (or 27 kDa), and the majority (30/37) were predicted to be secreted (Table 2).

The labels assigned to the 37 predicted LusCTLs are shown in Table 2. LusCTL1 and LusCTL2 were so named because they encoded proteins that were most similar to CTL1 and CTL2, respectively, which have been characterized in other species (e.g. A. thaliana [14] and G. hirsutum [10]) (Table 2). The gene LusCTL37

Figure 2. Phylogenetic relationships among LusCTLs. All predicted LusCTLs amino acid sequences were aligned using their deduced full-length peptide sequences in the MEGA platform (MUSCLE), Maximum Likelihood Method, JTT+G model, bootstrap 1000.

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Figure 3. Phylogenetic relationship among *LusCTL* and selected CTLs of 43 different plant species. All the predicted CTLs sequences were aligned using their deduced full-length peptide sequences in MEGA (MUSCLE), Maximum Likelihood Method, WAG+G model, bootstrap 1000. Classes I–IV were labeled according to Hamel et al. [21] and Zhang et al. [10] and Groups A–C are as shown in Figure 2. doi:10.1371/journal.pone.0097949.g003

Figure 4. Relative differential expression of *LusCTL* genes in different tissues of the flax stem. ΔΔC_{t} values for each of CTLs were generated using an average ΔC_{t} for all CTLs as a reference. Error bars show the standard error of the mean. doi:10.1371/journal.pone.0097949.g004
was predicted to encode only a protein of 69 aa, which is much shorter than the rest of the LusCTLs (Table 2), and so it was not used in further analyses.

The LusCTLs and their inferred phylogenetic relationships are shown in Figure 2. Based on this dendrogram, the predicted LusCTLs were divided into three groups: Group A included LusCTL1–6, Group B included LusCTL7–14, and Group C included LusCTL15–36 (Figure 2, Table 2). The flax-specific tree shown in Figure 2 was expanded by the addition of representative GH19 CTLs from other species (Figure 3). In this multispecies tree, LusCTLs of Group A, which includes LusCTL1 and LusCTL2, were part of the same clade as the well-characterized AICTL2 of *A. thaliana* and GhCTL1, GhCTL2, GhCTL7 of *G. hirsutum*. The Group B LusCTLs (LusCTL7–14) were in the same clade as the previously defined Classes I, II, III, GH19 chitinases [10,21]. Most of group B was in the same sub-clade as Class II, although none of the previously defined Classes I–III were monophyletic in our analysis. Finally, our Group C LusCTLs (LusCTL15–36) formed a monophyletic clade with representatives of the previously defined Class IV GH19 chitinases.

**LusCTL Transcript Expression**

Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed to study LusCTL expression patterns of *L. usitatissimum* genes of chitinase-like proteins in various tissues and stages of development (Table 1). These tissues and their names as used here are equivalent to the names used in previous studies [6,32,39,40]. Only 34 sets of primers were used in this assay, because members of each of two pairs of LusCTLs could not be distinguished by unique primers: LusCTL26 and LusCTL29 (95.6% aa and 96.3% nt identity), and LusCTL33 and LusCTL34 (99.6% aa and 98.8% nt identity). Thus a common set of primers was used for each of these pairs. We observed that transcripts of LusCTL1 showed enriched levels of expression (compared to the apical part of stem) in tissues in which cell walls were undergoing thickening (Figure 4) in xylem and in phloem fibers. Transcripts for this gene were enriched 57-fold in xylem, 28-fold in the MID region, and 20-fold in fiber. Another predicted CTL, LusCTL2, showed a similar pattern of enrichment in secondary-wall bearing tissues (8.3, 4.5 and 1.4-fold higher in xylem, MID and fiber, respectively, compared to the apex), although the magnitude of its enrichment was not as strong as LusCTL1. These two LusCTLs had high sequence similarity to each other (91.9% amino acid identity) and had similar patterns of expression as compared to each other in the various flax tissues.

A subset of LusCTL genes (LusCTL10, LusCTL11, LusCTL19, LusCTL20, LusCTL21, LusCTL23, LusCTL24, LusCTL26) had high relative expression in tissues that contained phloem fibers (MID, peel, fiber) but low relative expression in xylem (Figure 4). Three of these genes (LusCTL19, LusCTL20, LusCTL21) were enriched > 40-fold in fibers compared to the apical part of stem. These three genes had high similarity to one another (76% identity between LusCTL19 and LusCTL20 as well as between LusCTL19 and LusCTL21; 91% identity between LusCTL20 and LusCTL21) (Figure 2).

**LusCESA Phylogenetic and Expression Characterization**

To provide context for the expression patterns of LusCTLs, and to test whether the expression pattern of cellulose synthase (LusCESA) genes differed between gelatinous fibers and cells with a xylan type of secondary cell wall, expression of LusCESA in different flax tissues was analyzed. We identified 14 predicted LusCESA genes in the flax whole genome assembly by searching predicted proteins for the conserved cellulose synthase domain (Pfam PF03552). No putative LusCESA7 genes were found in the original published genome published (v1.0, [31]). However, though BLAST alignment of the CDS of poplar *LusCESA* isoforms identified, and these were annotated using the Augustus server (http://bioinf.uni-greifswald.de/augustus/). Thus, all 16 predicted LusCESA genes were aligned with well-characterized *AtCESA* genes from *A. thaliana* and PtiCESA from *P. trichocarpa* (Figure 5). This alignment was used to construct a phylogenetic tree and annotate the LusCESA, which were named according to the established *A. thaliana* [41] and *P. trichocarpa* nomenclature systems (Table 3, [33]). The number of LusCESA and PtiCESA isoforms identified for each of the eight major types of CESAs was similar except in...
the case of CESA3, where one more gene was identified in *P. trichocarpa* than in *L. usitatissimum* (Figure 5, Table 3). The *LusCESA* appeared to be typical of other genes in this family in that they were large integral membrane proteins with eight predicted transmembrane domains, a hydrophilic domain that faces the cytosol, and a zinc finger domain at the N-terminus of proteins with the characteristic amino acid motif “CxxC” (specific for CESAs only [42]).

Relative differential expression of *LusCESA* genes in different tissues of the flax stem was estimated (Figure 6). *LusCESA4*, *LusCESA8-A*, *LusCESA8-B*, *LusCESA7-A*, *LusCESA7-B* had high expression in tissue that produce secondary walls (TOP, MID, Xylem, Fiber, Root). Transcripts of these *LusCESA* isoforms were the most enriched in Xylem, which contained cells with xylan-type cell walls, and in roots, where secondary vascular tissue (xylem) was also well-developed. These secondary cell wall type *LusCESAs* had also high relative expression in cellulosic fibers, although it was not as strong as for xylem.

Changes in expression of the *LusCESA* 4, 7, 8 isoforms and “xylem-specific” *LusCTL*1 and *LusCTL*2 were well-correlated in different flax tissues (Figure 7A). This group of genes was highly expressed in tissues with secondary cell walls (MID, Xylem and Roots). In contrast, the “fiber-specific” *LusCTLs* had very different patterns of expression in the same tissues (Figure 7B): these had low level of expression in xylem, but high level of relative expression in tissues with gelatinous fibers (peel and fiber).

**Discussion**

Certain fibers of many plant species form G-type cell walls, which are rich in crystalline cellulose [1]. Expression of CTLs has been previously reported to be enriched during development of G-type cell walls, along with specific FLAs [4,6,7], LTPs [6] and BGALs [6–8]. In this work, we analyzed expression of all *LusCTL* genes of GH 19 in different flax tissues and compared this expression with *LusCESAs* and to their inferred phylogenies.

![Figure 6. Relative expression of *LusCESAs* genes in different tissues of the flax stem.](https://www.plosone.org/doi/fig/10.1371/journal.pone.0097949.g006)
In the flax genome, 16 predicted *LusCESA* were identified (Table 3). Previously only partial sequences of some flax *CESA* were published [43]. All 16 flax *CESA* could be placed in discrete clades with *Arabidopsis* and *Populus* *CESA* homologs (Figure 5). We generally numbered *LusCESA* in a way that reflects the association of each flax gene with its nearest relative in the *Arabidopsis* genome, as was done for *CESAs* of *Populus* [33]. Following this pattern, the *LusCESA6A–F* genes we named as a group, similar to *PtCESA6A–F* and were not distinguished as *CESA12/9/5/6* as in *Arabidopsis* clade (Table 3) [44]. Most of the flax and *Populus* *CESA* genes are present as pairs of paralogs in their respective genomes, although there were three *LusCESA3* genes (*LusCESA3A–C*) for only two *Populus* genes and one *Arabidopsis* gene. *AtCESA10*, *AtCESA1* and *AtCESA10* were represented by only one pair of genes (*LusCESA1A, B*) in flax.

It is well established that proteins encoded by different sets of three *CESA* genes (*CESA1, 3, 6* and *CESA4, 7, 8*) are required for cellulose synthesis during primary and secondary wall formation, respectively [44–46]. The functional relationships of the various paralogs of *LusCESA* (except *LusCESA4*, Table 3) are presently unclear. According to the data obtained here, secondary cell wall *LusCESA4*, *LusCESA7-A, B* and *LusCESA8-A, B* were highly expressed both in the xylem cells with lignified cell walls (i.e. xylan type) and in the phloem fibers with thick gelatinous cell wall. This suggests that phloem fibers and xylem may use similar, rather than specialized rosettes. This is consistent with observations from poplar showing only minor differences in expression of cellulose biosynthetic genes in tension wood as compared to normal wood [3]. The different properties of gelatinous and xylan type cell walls are therefore likely determined not by *CESAs*, but by other proteins associated with cellulose synthesis, which could include specific CTLs.

We observed two *LusCTLs* that were expressed more strongly in xylem tissue than in any other tissue surveyed (Figure 3, *LusCTL1, LusCTL2*). The co-expression of certain isoforms of *LusCTL1, LusCTL2* and the secondary wall *LusCESA* (*CESA4, 7, 8*) suggested a role for these *LusCTLs* in secondary cell wall development (Figure 7). As noted above, *LusCTL1* and *2* are highly similar to *AtCTL1* of *A. thaliana* and *GhCTL1, GhCTL2*, of *G. hirsutum*. The role of *CTL2*, and its close homolog *CTL1*, in cell wall biosynthesis is especially intriguing since associations between CTLs and primary or secondary cell wall synthesis have been reported in different plant species [10]. *CTL2* is strongly upregulated during secondary wall formation in interfascicular fibers in *A. thaliana*. Reduction in crystalline cellulose content in *ctl1 ctl2* mutants was demonstrated, leading to the to the suggestion that *AtCTLs* are involved in cellulose assembly. Furthermore, in *P. trichocarpa*, expression of chitinase genes related to *AtCTL1, AtCTL2*, and *GhCTLVII* are highly correlated with secondary wall formation of xylem [47]. It has therefore been proposed that *CTL1* and *CTL2* work in conjunction with primary- and secondary-cell wall *CESAs*, respectively [14]. One of the hypotheses for *CTL1/2* function is regulation of cellulose assembly and of interaction with hemicelluloses via binding to emerging cellulose microfibrils [14]. However, the mechanism of *CTL* action in cell wall biosynthesis as well as substrates of catalytic activity (if any) remains unknown. It was suggested that the likely substrates of plant chitinases may be arabino/galactan proteins, chitooligosaccharides and other GlcNAc-containing glycoproteins or glycolipids [13,15,48,49] and the mechanism by which CTLs act is more likely to involve binding of chitin oligosaccharides than catalysis. Also, it is assumed that chitinases may participate in the generation of such signal molecules that regulate the organogenesis process [50].

Although relative expression of *LusCESA* (*4, 7, 8*) and *LusCTL1, LusCTL2* in xylem tissue was higher compared with phloem fibers, we cannot exclude involvement of these *LusCTLs* in phloem fiber cell wall development. At the same time, a distinct group of *LusCTLs* (*LusCTL19, LusCTL20, LusCTL21*) had very high enrichment in samples with phloem fibers (MID, peel, fiber) with a low level of expression in xylem. According to the phylogenetic tree, these *LusCTLs* (group C) were most similar to the previously defined Class IV chitinases (Figure 4). High constitutive expression of Class IV (along with Class I) in most organs of *A. thaliana* under normal growth conditions has been previously noted [51]. Detailed bioinformatic characterization of genes of *LusCTL* distinct group should be conducted in future. Probably *LusCTLs* that are highly expressed in fibers may be specific to the gelatinous cell wall, while *LusCTL1* and *LusCTL2* are essential for wall thickening in general.
Conclusion

High expression of specific LusCTLs was observed in different types of thick cell wall producing tissues. LusCTL1 and LusCTL2 were preferentially expressed during secondary wall deposition of xylem and were coexpressed with secondary cell wall CESAs (4, 7, 8). Another group of LusCTLs, especially LusCTL19, LusCTL20, LusCTL21 were highly expressed in bast fibres, which have cellulose-rich, gelatinous walls. The group of fiber-enriched LusCTLs was expanded in flax compared to species that do not produce bast fibres, suggesting that these genes might play a unique role during gelatinous cell wall development in general and cellulose synthesis in particular. It is possible that the presence of fiber-specific LusCTLs, along with other key participants, determines differences in mechanisms of xylan and gelatinous cell wall formation. To establish the functions of these LusCTLs further characterization, including analysis of enzyme activity and structure, is necessary. Chitinase-like proteins remain one of the most mysterious proteins in the plant cell wall. This study provides further evidence of their involvement in the process, and distinguishes between groups of CTLs involved in different type of cell wall development.

Supporting Information

File S1 LusCTL gene sequences. (XLSX)

Author Contributions

Conceived and designed the experiments: NM TG MKD. Performed the experiments: NM. Analyzed the data: NM TG MKD. Contributed reagents/materials/analysis tools: NM TG MKD. Wrote the paper: NM TG MKD.

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