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A Cell Wall Proteome and Targeted Cell Wall Analyses Provide Novel Information on Hemicellulose Metabolism in Flax*

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Experimentally-generated (nanoLC-MS/MS) proteomic analyses of four different flax organs/tissues (inner-stem, outer-stem, leaves and roots) enriched in proteins from 3 different sub-compartments (soluble-, membrane-, and cell wall-proteins) was combined with publicly available data on flax seed and whole-stem proteins to generate a cell wall protein database containing 2996 nonredundant total proteins. Subsequent multiple analyses (MapMan, CAZy, WallProtDB and expert curation) of this database were then used to identify a flax cell wall proteome consisting of 456 nonredundant proteins localized in the cell wall and/or associated with cell wall biosynthesis, remodeling and other cell wall related processes. Examination of the proteins present in different flax organs/tissues provided a detailed overview of cell wall metabolism and highlighted the importance of hemicellulose and pectin remodeling in stem tissues. Phylogenetic analyses of proteins in the cell wall proteome revealed an important paralogy in the class IIIA xyloglucan endo-transglycosylase/hydrolase (XTH) family associated with xyloglucan endo-hydrolase activity.

Flax (Linum usitatissimum L.) is an economically important species that is grown for its cellulose-rich bast fibers used in textiles (linen) and composites, as well as for its seeds that are used as animal feed and a source of oils containing unsaturated fatty acids such as Alpha linolenic acid (ALA) (1). Flax seeds also contain high amounts of the lignan secoisolarici-resin diglucoside (SDG) that shows several biological activities of interest for human health (2).

As in other plant species improvement in the production and quality of products obtained from flax, as well as a better tolerance to abiotic stress and pathogens requires a more thorough understanding of many aspects of flax biology. Over the last decade a number of genomics, transcriptomics and functional studies have made significant contributions to our knowledge on flax, but proteomic studies have been generally limited to certain organs and/or cell types and a more comprehensive overview of the flax proteome is as yet lacking (3–13). This represents an important gap in our knowledge because although transcriptomics, for example, provides information about transcript accumulation in a biological sample, other factors (e.g. translation efficiency, protein turnover, post-translational modifications etc.) can also affect the final quantity and biological activity of the corresponding protein (14, 15). Proteomics is therefore an important complementary tool that has been successfully used in plant biology to improve our understanding of many different processes (16, 17).

Given their economic interest, most proteomics studies in flax have focused on stems and seeds (11–13, 18). In the case of stems, the interest is mainly directed toward the cellulose-
rich bast fibers that are in the outer stem tissues. Nevertheless, the inner stem tissues (shives) are not without interest as they represent a potentially interesting by-product of bast fiber extraction that can be used for composites, animal bedding and bioenergy. In both cases industrial quality is closely related to the structure of the corresponding plant cell walls that are produced and modified during the life of the plant via the action of a complex battery of enzymes involved in the biosynthesis and the remodeling of different cell wall polymers (e.g. cellulose, hemicelluloses, pectin, lignin). An improved knowledge of these enzymes, as well as of other structural (nonenzymatic) cell wall proteins, will contribute to a better understanding of how cell wall assembly (and hence quality) is regulated in fibers and shives.

In a previous proteomics study of the whole flax stem (i.e. inner-and outer-stem tissues NOT separated) we identified 152 cell wall proteins based on the use of TargetP, Predicator, and WoLF PSORT algorithms (13). Although this work provided an interesting insight into flax stem cell wall metabolism it did not consider the very different structures of cell walls from stem inner- and outer-tissues (19, 20). The fiber cell wall has been extensively investigated by different authors and is characterized by an extremely thick secondary layer that is sometimes referred to as a gelatinous layer (G-layer) because of the similarity with the corresponding layer in tension wood G-fibers (21–23, 19). Analyses of the flax fiber cell wall shows that it is made up of ~70% cellulose, 5–15% noncellulosic polysaccharides (NCPs) consisting of beta-1,4-galactans and arabinogalactans, as well as extremely low amounts of lignin (20, 24–26). In contrast, the walls of xylem (shive) cells contain lower amounts of cellulose and much higher amounts (approx. 30%) of lignin (20). Because cell wall structure is the result of the combined action of cell wall biosynthesis and remodeling enzymes it is of interest to compare the cell wall proteomes of these two contrasted tissues. Further information contributing to a better understanding of the link between cell wall structure and the cell wall proteome can also be obtained by analyzing other organs that contain different/similar cell types (e.g. leaves that contain a majority of cells with primary cell walls and roots that have a similar structure to whole stems).

Even though cell wall polymers are localized in the cell wall, many of the proteins associated with their biosynthesis are not localized in this compartment. For example, the phenylpropanoid enzymes involved in lignin monomer (monolignol) biosynthesis are localized in the cytosol and the cellulose synthase enzymes are associated with the cellulose synthase complex (CSC) in the plasma membrane (27, 28). Similarly, glycosyltransferases (GTs) associated with hemicellulose and pectin biosynthesis are located in Golgi membranes (29, 30). In contrast, glycosylhydrolases (GHs) involved in cell wall remodeling are indeed located in the cell wall (31). A global view of the ‘cell wall proteome’ should therefore consider not only those proteins that are physically located in the cell wall, but also relevant proteins that are present in other compartments. We therefore used protein extractions that were designed at producing fractions enriched in membrane proteins, as well as soluble and cell wall proteins (21, 33, 34).

Altogether our results represent an important contribution to our understanding of cell wall biology in flax. This knowledge will provide a clearer vision of bast fiber construction and the link between cell wall structure and quality, not only in flax, but also in other commercially important species such as hemp and jute. In addition, a better understanding of the molecular mechanisms that enable plant cells to build celllose-rich walls will provide fundamental knowledge necessary to engineer plant species for the production of more efficient lignocellulosic biomass for biofuels.

**EXPERIMENTAL PROCEDURES**

**Plant Growth**—Seeds of the flax (*Linum usitatissimum* L.) cultivar Diane were germinated on moistened paper for 24 h at 25 °C in the dark, and then transferred to hydroponic culture and grown for a total of 70 d (corresponding to the vegetative stage of growth when cell wall formation is active) on increasing concentrations of M&S nutrient solution in the absence of sucrose and agar according to the following regime (32, 33). Days 0–30 (1/20 M&S renewed every 2 d, photoperiod 12/11 h day/night, temperature 14/12 °C day/night); days 30–60 (1/15 M&S renewed every 1 d, photoperiod 13/11 h day/night, temperature 16/14 °C day/night); days 60–70 (1/10 M&S renewed every 1 d, photoperiod 14/10 h day/night, temperature 18/16 °C day/night). Light intensity was 210,18 μmol/s/m², and hygrometry was 80%.

Leaves, roots and stems were harvested separately after 70 d and rapidly frozen in liquid nitrogen, and stored at −80 °C. For the stems, outer tissues and inner tissues were separated as described previously (20).

**Experimental Design and Statistical Rationale**—Leaves, roots, outer stems, and inner stems from 40 plants were ground independently in liquid nitrogen to a fine homogenized powder using a grinder and then pooled to constitute 2 biological replicates, each consisting of material from 20 plants. Two g powder were used for the extraction of soluble and cell wall proteins and 7 g were used to obtain a fraction enriched in membrane proteins. Altogether 24 samples were obtained: 4 organs/tissues × 3 sub-fractions × 2 biological replicates. Two technical replicates were performed for each biological replicate. Only proteins identified in both biological replicates were retained for further analyses.
**Flax Cell Wall Proteome**

**Soluble (S) and Cell Wall (C) Protein Enrichment—**Soluble proteins were extracted according as previously described (34). Briefly, 2 g ground sample were further ground for 5 min in 10 ml Tris-HCl buffer (50 mM, 0.06% PIC (w/v), Protease inhibitor mixture), pH 7.5; Sigma-Aldrich) and then centrifuged (10 min at 4 °C, 16,000 × g). The pellet was recovered and stored at −80 °C for cell wall protein (CWP) extraction (see below). The supernatant was recovered and incubated (15 min, room temperature, gentle agitation) with protease inhibited (1 mg/ml) before being centrifuged (10 min, 18,000 × g). The resulting supernatant was recovered and soluble proteins (S) were precipitated by incubation with 72% TCA (w/v, trichloroacetic acid, 1 h, −20 °C). The pellet was washed once with cold acetone and dried at room temperature (5 min) before being stored at −20 °C.

CWPs were extracted from the CWP pellet as previously described (35). Briefly, the pellet was washed with 500 ml sodium acetate buffer (5 mM, pH 4.6) and filtered through a nylon membrane (40 × 40 microns, Millipore Corporation, Bedford, MA). The pellet was washed again with sodium acetate buffer (5 mM, pH 4.6) and then incubated with 20 ml 1.5 M NaCl (30 min at 4 °C with gentle agitation). The solid cell wall residue was then further extracted by (1) incubation with 20 ml CaCl2 buffer (5 mM sodium acetate, 200 mM CaCl2, 30 min at 4 °C) and (2) incubation with 20 ml LiCl buffer (5 mM sodium acetate, 2 M LiCl, 30 min at 4 °C). The liquid fractions from all CWP extractions were combined and CWPs precipitated by adding 10% TCA (w/v).

**Membrane (M) Protein Enrichment—**Membrane (M) proteins were extracted from 7 g ground sample according to Song et al. (58). Briefly, a small amount of 1% PVPP (polyvinylpyrrolidone) was added to ground material before being incubated in 50 ml extraction buffer (0.5 mM Tris-HCl, pH 8.5, 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 2% (v/v) β-mercaptoethanol, 0.1% w/v PIC), 5 min at 4 °C). Next, the homogenate was centrifuged (10 min, 12,000 × g, 4 °C) and filtered. The liquid fraction was diluted by an equal volume of ice-cold water and centrifuged (150,000 × g, 40 min). The resulting pellet was then washed three times with ice-cold water and dissolved in SDS buffer (0.5 mM Tris-HCl pH 8.5, 2% (v/v) β-mercaptoethanol, 30% (v/v) glycerol, 4% SDS, 1 mM PMSF, 0.1% PIC) and heated for 5 min at 80 °C, before being centrifuged (12,000 × g 30 min at room temperature). The supernatant was extracted three times with an equal volume of water-saturated phenol. Proteins were precipitated at −20 °C overnight from the phenol phase by adding 5 volumes of cold methanol containing 0.1 M ammonium acetate. After precipitation, the proteins were pelleted by centrifugation (12,000 × g, 10 min at 4 °C) and washed three times with 90% cold methanol, and once with 90% cold acetone before being vacuum dried (5 min).

Samples were subsequently prepared for analyses by suspension in rehydration buffer (7 M Urea, 2 M Thiourea, 100 mM DTT, 2% w/v CHAPS). Protein content was determined using a reducing agent (BioRad) and a detergent compatible (Bradford) assay.

Twenty-five μg total proteins per sample were partially separated by electrophoresis on a precast ready gel according to the manufacturer’s instructions (Criterion™ XT precast 1D gel, 4–12% Bis-Tris, 1 mm x 12 wells, Bio-Rad). The gel was stained with Instant Blue dye (Gentaur BVBA, Kampenhout, Belgium) and 5 bands were excised from each sample lane, cut into 1–2 mm cubes and transferred into a microplate. Proteins were reduced with 10 mM DTT (in 100 mM ammonium bicarbonate) for 30 min at 56 °C, then alkylated with 55 mM Iodoacetamide (in 100 mM ammonium bicarbonate) for 20 min at room temperature. Finally, gel pieces were de-stained and then digested overnight by modified trypsin enzyme (sequencing mass grade, Promega). Peptides were recovered and separated on a NanoLC™-2D System (Eksigent, Sciex, Belgium) coupled to a TripleTOF® 5600+ mass spectrometer (Sciex, Belgium). Peptide desalting and enriching were achieved using a pre-column (C18 Pep-Map™, 5 μm, 5 mm × 300 μm i.d., Thermo scientific, Bremen, Germany). Peptides were separated and eluted on a C18 reverse phase column (PepMap™ 100, 3 μm, 100Å, 75 μm i.d. × 15 cm, Thermo scientific) using a linear binary gradient (solvent A: 0.1% FA (formic acid); solvent B: 80% ACN 0.1% FA; 5 min 5% B, 40 min 5% to 55% B, flow rate of 300 nl/min). The peptides were injected into the TripleTOF® 5600+ with a NanoSpray III source using a 10 μM i.d. emitter (New Objective, Woburn, MA). The source parameters used vary depending on optimized conditions on each day, the values were for gas1 = [−1–6], gas2 = 0, curtain gas = [20–30], the ion spray voltage −2.2keV. For each sample 2 biological and 2 technical replicates were randomly analyzed.

MS analysis was performed in information-dependent acquisition mode. MS spectra were acquired using 250 ms accumulation time per spectrum with a mass range of 300–1250 Da. The top 20 precursor ions were selected in each MS scan for subsequent MS/MS scans with high sensitivity during 250 ms of accumulation time (range from m/z 100–1250 Da) and the voltage was automatically adjusted with the system of rolling collision energy. The dynamic exclusion was set at 10 s. Systems were controlled by Analyst software (version TF1.7, Sciex). An automatic mass recalibration was performed using a beta-galactosidase digest during the sequence of samples.

CID spectra were processed by Mascot (version 2.4.2) using Protein Pilot (version 4.5, Sciex) by searching against the Linum usitatissimum database (v1.0, http://www.phytozone.net released on 10th December 2014, 43,484 entries). The searches were performed with the following parameters: enzyme: trypsin, 2 missed cleavages, mass accuracy precursor: 20 ppm, mass accuracy fragments: 0.3 Da, fixed modifications: Carboxymethyl (C), dynamic modifications: Oxidation (W), Oxidation (HW), Trp->Kynurenin (W), Oxidation (M). Three supplemental filters were applied to Mascot results: (1) a peptide confidence (p > 0.05), (2) an individual ion score (calculated by Mascot) of the considered research, and (3) a minimum of two significant peptides per protein. Only proteins fulfilling these criteria were retained. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (36) partner repository (ProteomeXchange title: Flax cell wall proteome; ProteomeXchange accession PXD005003; http://www.ebi.ac.uk/pride/archive/projects/PXD005003; PubMed ID: 28706005).

**Protein Identification and Functional Classification—**Flax proteins were identified directly via the inclusion in the Mascot database of peptides sequences based on the sequenced Linum usitatissimum genome in Phytozone (http://www.phytozone.net). Only proteins present in both biological repetitions independently run by MS analysis were retained for further study. The predicted protein annotation was checked and completed with InterPro v.48 (http://www.ebi.ac.uk/interpro/) and CAZy databases (http://www.cazy.org/). Functional classification was performed using MapMan (http://mapman.gabipd.org/web/guest/mapmanstore) (37). Subcellular locations were predicted using Predotar v. 1.03 (https://urgi.versailles.inra.fr/Tools/Predotar), TargetP1.1 (www.cbs.dtu.dk/services/TargetP/), and SignalP 4.1. (www.cbs.dtu.dk/services/SignalP/) (38). Annotation of enzymes that assemble, modify, and breakdown oligo- and polysaccharides was done according to the CAZy database (39) and the scientific literature. Peroxidases were named according to the Peroxibase (40). The predicted cell wall location of proteins was verified using WallProt database (www.polebio.isrv.ups-tlse.fr/WallProtDB) and proteins were functionally classified according to classes established in this database.

**Phylogenetic Analysis—**Homologs of Linum usitatissimum CWPs were selected by BLASTp, (e-value cutoff of 1e-20, percentage coverage ≥ 50% minimum, maximum of 1000 sequences) against a database of selected proteomes from phytozone v.10 (L. usitatissimum, P. trichocarpa, M. truncatula, R. communis, G. max, P. persica,
A. thaliana, B. distachyon, P. patens, C. reinhardtii, V. carteri f. naga-
riensis, M. pusilla CCMP1545, O. lucimarinus, M. esculenta, A. coerus-
lea, C. sinensis, E. grandis, G. raimondii, S. purpurea, V. vinifera, P. 
virgatum. The number of taxa was limited to present an easily un-
derstandable tree that: (1) illustrates plant diversity, (2) includes the two 
Angiosperm model plants (A. thaliana, B. distachyon), and (3) presents 
all the L. usitatissimum sister taxa (P. trichocarpa, M. esculenta, R. 
communis, S. purpurea). The selected set of homologous sequences 
was aligned using multiple sequence comparison by log 
expectation (MUSCLE) (Edgar, 2004). Block selection was performed with 
BMGE (Criscuolo and Gribaldo 2010). Reduced alignments were 
analyzed under maximum likelihood with the LG4X (Le et al., 2012) 
and 1000 bootstrap repetitions with IQ-TREE software (Nguyen et al., 
2015).

Preparation of Flax Hemicellulose Fraction—Alcohol-insoluble cell 
wall residue (AIR) was prepared by grinding 5 g inner- and outer-stem 
tissues in liquid N2 and extracting in 20 ml 70% EtOH (V/V) for 15 min 
at 70 °C prior to centrifugation (10 min, 5000 × g) and pellet recu-
tation. This step was repeated 3 times and the pellet (AIR) recov-
ered and lyophilized (Fry, 1988). A hemicellulose fraction was ob-
tained by sequential extraction. Pectins were first removed from AIR 
by heating (100 °C, 2 h) in ammonium oxalate (0.5% P/V) followed by 
centrifugation, elimination of the supernatant and washing of the 
pellet (>2) in 20 ml 4 M NaOH. This step was repeated twice and the 
pellet was then incubated overnight in 4 M NaOH at 4 °C to extract hemicelluloses. The basic extract was then neutralized by 6N HCl, 
centrifuged (10 min, 5000 × g) and the supernatant recovered and 
dialyzed twice before being lyophilized.

Generation of Oligo-hemicelluloses—The lyophilized fraction contain-
ing flax hemicelluloses was dissolved in 2.5 ml ammonium carbonate 
(10 mm, pH 5.5) and 0.5 ml digested by either (1) 2.5 units of endo-
β-glucanase (Megayme) for 18 h at 37 °C to generate oligo-xyllo-
glucans or (2) 1 unit of endoxylanase (Megazyme) for 18 h at 28 °C 
to generate oligo-xylans. The digestions were stopped by adding 3 
 volumes 95% EtOH and the liquid phase (containing the oligo-xylo-
glucans/xyllans) lyophilized and then dissolved in ultra-pure water.

MALDI-TOF-MS—One microoliter of oligo-xylolglucans/xyllans was 
cocrystallized with 1 μl 2,5 dihydrobenzoic acid prepared by dissolv-
ing 5 mg DHB in 500 μl acetoniitric acid/0.1% TFA (70:30, V/V) on 
 a stainless steel support. Mass spectra were recorded on a TOF E MS 
(Micromass, Manchester, UK) in positive mode. Spectra were cali-
brated using the Sequazyme Peptide Mass Standards Kit (Applied 
 Biosystems).

Light Microscopy—Flax stem samples were excised at 10 cm 
above the insertion point of cotyledon and fixed in a formaldehyde: 
 acetic acid: ethanol solution (FAE, 3:5:6.5:90, v/v,) , progressively de-
hydrated in an alcohol series and infiltrated with Technovit 7100 resin 
(Kulzer), Sections (3 μm) were cut using a Leica RM 2065 microtone 
and stained with a 0.5% (w/v) aqueous solution of Toluidine Blue O 
(TBO) (Sigma) for examining tissue organization. Sections were ex-
amined with a Leica DM2000 microscope coupled to a Leica DFC320 
camera. Images were analyzed with the Leica Application Suite 
program.

Immunolocalization—Resin was removed by acetone, and sections 
were rehydrated in a 5 min graded ethanol series followed by immer-
sion in PBS. For immunolocalization, nonspecific sites were saturated 
in 2 × 15 min PBST (2% BSA (w/v) and 0.05% tween 20 (v/v) in PBS) 
followed by immunolocalization of xylan epitopes with rat monoclonal 
LM10–11, xylolglucan epitopes with LM-15–24 and mannan/hetero-
mannan with LM21 primary antibodies (Plant Probes, Leeds, U.K.). 
Experiments were performed on semi-thin sections (5 μm), using 
diluted 1/10 antibodies in a PBST buffer (137 mM NaCl, 2.7 mM KCl, 
16.3 mM Na2HPO4, 1.5 mMKH2PO4, pH 6.8, 2% (w/v) BSA, 0.01% 
(v/v) Triton X-100). After rinsing in three 10 min changes of PBS at

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Inclusion of previously published large-scale proteomics data on flax seeds (1516 proteins) and whole stems (1135 proteins) allowed us to recover 1072 (seeds), 550 (whole stems) and 132 (seeds and whole stems) nonredundant additional proteins bringing the total of nonredundant flax proteins to 2996 (supplemental Data S3, supplemental Fig. S2) (12, 13). The raw data and the results of the database searches corresponding to these analyses are publicly accessible via ProteomeXchange (http://www.ebi.ac.uk/pride/archive/projects/PXD005003) with identifier PXD005003.

To obtain a global view of the biological processes in which the identified flax proteins were involved we classified them into different MapMan Bins (Fig. 2) (http://mapman.gabipd.org/web/guest/mapmanstore) (37). The class ‘proteins’ was particularly highly represented in all samples with more than 21% total proteins, followed by the class “unassigned.” Specific organs were particularly rich in certain categories (e.g., the class “photosynthesis” in leaves; the class “lipid metabolism” in seeds, or the class “miscellaneous” in roots) reflecting the biological specificity (e.g., leaves, seeds) and/or our lack of functional knowledge (e.g., roots). Forty proteins were present in all 6 organs and as could be expected, are involved in biochemical processes common to most cell types such as protein synthesis and energy production as previously observed for the “mitochondrial ATP synthase D chain” and “H+-ATPase” proteins in Arabidopsis (42).

Flax Cell Wall Proteome Compilation and Organ-/Tissue-Specific Analysis of Selected Processes—To create the flax cell wall proteome we selected proteins associated with cell wall metabolism and/or physically localized in the cell wall according to a number of different criteria. Plant cell walls contain high amounts of polysaccharides (cellulose, hemicelluloses, pectins) and we first analyzed our data set to identify different CAZy (Carbohydrate Active Enzymes) families that are involved in the biosynthesis and remodeling of carbohydrates (39). Overall 207 CAZy proteins are distributed among the 7 CAZy classes: glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), auxiliary activities (AA), carbohydrate binding modules (CBM) and expansin (Expn) (supplemental Fig. S3, supplemental Data S4). Analyses of CAZy proteins from combined organ/tissue data indicated that GH was the most represented class (39%), followed by AA (30%), GT (16%), CE (9%), CBM (4%), and 1% (PL, Expn).

Subsequent examination of individual families within each CAZy class allowed the identification of 178 proteins potentially associated with a wide range of cell wall enzymatic activities and/or actions on cell wall structure (Table I, supplemental Fig. S4). These activities can be divided into 2 main types of action: (1) biosynthesis of cell wall polymers (GT and AA CAZy families) and (2) remodeling of cell wall polymers (GH, CE, PL, CBM, EXPN families). For polymer biosynthesis, 8 different cell wall GT families are represented including GT2 (cellulose), GT4 (cellulose - sucrose synthase), and GT75 (GAX) (43). Other plant GT families involved in hemicellulose (GT47, GT8), callose (GT48) and pectin (GT8) biosynthesis are also represented. Another highly represented family includes the GT1 family that contains UDP-glycosyltransferases (UGTs) glycosylating secondary metabolites and hormones and that may be involved in regulating lignin monomer availability (44, 45). In the class AA, 6 families were present including the AA2 family containing peroxidases and the AA1 family containing laccase enzymes involved in lignin biosynthesis and cell wall reticulation. For polymer remodeling, 14 different cell wall GH families and 2 CBM families are present with potential actions on hemicelluloses (GH1, GH3, GH5, GH9, GH10, GH16, GH31, GH51, and GH95) and pectins (GH28 and GH35) being the most represented (31). Families responsible for pectin acetylation (CE13) (46), methylesterification (CE8) and hydrolysis (PL4) are also represented as are 1 EXPN family (cellulose-hemicellulose interaction).

In addition to polysaccharides, plant cell walls in certain tissues (e.g., xylem, sclerenchyma) also contain large amounts of lignin. We therefore analyzed our data set to identify proteins involved in lignin metabolism to add them to the flax cell wall proteome. To obtain a global view of the biological processes in which the identified flax proteins were involved we classified them into different MapMan Bins (Fig. 2) (http://mapman.gabipd.org/web/guest/mapmanstore) (37). The class ‘proteins’ was particularly highly represented in all samples with more than 21% total proteins, followed by the class “unassigned.” Specific organs were particularly rich in certain categories (e.g., the class “photosynthesis” in leaves; the class “lipid metabolism” in seeds, or the class “miscellaneous” in roots) reflecting the biological specificity (e.g., leaves, seeds) and/or our lack of functional knowledge (e.g., roots). Forty proteins were present in all 6 organs and as could be expected, are involved in biochemical processes common to most cell types such as protein synthesis and energy production as previously observed for the “mitochondrial ATP synthase D chain” and “H+-ATPase” proteins in Arabidopsis (42).

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In addition to polysaccharides, plant cell walls in certain tissues (e.g., xylem, sclerenchyma) also contain large amounts of lignin. We therefore analyzed our data set to identify proteins involved in lignin metabolism to add them to the flax cell wall proteome.
Our results (supplemental Fig. S5, supplemental Data S5) indicate that 24 proteins belonging to 9 out of the 11 canonical monolignol biosynthesis enzyme families are present in different flax samples. Only CSE (Caffeoyl shikimate esterase) and F5H (ferulic acid/coniferaldehyde 5-hydroxylase) proteins were not detected. Different individual proteins of the same family could be present in the same or different organs/tissues except for leaf samples where no monolignol biosynthesis enzymes were detected. Lignin formation occurs by a 2-step oxidative polymerization process in which monolignols are first oxidized to highly reactive radicals by laccases and/or peroxidases before undergoing spontaneous polymerization (27). Flax peroxidases and a laccase potentially involved in this process are indicated in supplemental Data S5.

Together with enzymes involved in the biosynthesis/remodeling of different cell wall polymers, cell walls also contain other non-CAZy proteins that are involved in the structuration of the cell wall as well as in other important biological roles such as defense and signaling. We therefore identified and added these proteins to the cell wall proteome set. A general overview of their broad biological roles was achieved by grouping proteins into 9 functional classes according to WallProtDB (http://www.polebio.lrsv.ups-tlse.fr/WallProtDB) (47) (supplemental Fig. S6, supplemental Data S6). Global analyses of pooled data from all organs/tissues showed that the largest class corresponded to ‘proteins with unknown functions’ (UF, 25%) highlighting our current lack of knowledge about plant secreted proteins. Other classes included “proteins acting on cell wall polysaccharides” (PAC, 19%), “miscellaneous” (M, 13%); “signaling” (S, 13%), “oxidoreductases” (OR, 12%), “proteases” (P, 12%), “proteins with interacting domains” (ID, 3%), “lipid metabolism” (LM, 3%), and “structural proteins” (SP, <1%).

Finally, we also added proteins identified in the MapMan cell wall bin (Fig. 2, supplemental Data S7), but not included in the groups discussed above. This group contains mainly enzymes involved in the synthesis/inter-conversion of UDP-activated cell wall polymer sugars, as well as a member of the COBRA family of proteins related to cellulose production (48). Interestingly, a β-1,4 glucanase protein belonging to the GH3 family, but not identified in the CAZy group was also present, as was an LRR protein.

Altogether the flax cell wall proteome contained 465 nonredundant proteins corresponding to: (1) cell wall related CAZy proteins, (2) lignin biosynthesis enzymes, (3) secreted proteins, and (4) other MapMan cell wall bin proteins identified in
4 different flax organs and 2 stem tissues (Fig. 3, supplemental Data S8, supplemental Fig. S7). We then determined the number of identified proteins and protein families involved in (1) the biosynthesis and remodeling of the different major cell wall polymers (Fig. 4) and (2) cell wall structuration/growth and defense (Fig. 5). Overall, the relative abundance of different proteins and protein families in the different samples appeared to reflect many organ-/tissue-specific differences in cell wall biology. These results are further discussed below (Discussion).

**XTH Paralogy and Characterization of Flax Stem Hemicelluloses**—Our results showed that proteins associated with hemicellulose and pectin metabolism are well represented in the flax stem cell wall proteome. Although recent studies (5) have contributed to a better understanding of pectin (β-1,4-galactan) metabolism in flax stems, hemicelluloses have received less attention despite their likely central importance in both load-bearing and cell expansion processes (49, 50, 51, 52). To learn more about the global potential of hemicellulose metabolism in flax we generated maximum likelihood phylogenetic trees (data not shown) for all “hemicellulose-related proteins” identified in the cell wall proteome. Inspection of the different trees allowed us to identify 2 protein families showing

**Table I**

List of CAZy families involved in cell wall polysaccharide, (glyco)protein and lignin metabolism identified in flax proteome. Families marked with an asterisk are potentially involved in hemicellulose metabolism. Numbers in brackets after family indicate number of independent proteins identified. References: 1) Minic et al., 2008; 2) Frankova et al., 2014; 3) Derba-Maceluch et al., 2015; 4) Knoch et al., 2014; 5) Lim et al., 2001; 6) McFarlaine et al., 2014; 7) (29) 8) Pauly et al., 2013; 9) Atmojodo et al., 2013; 10) (43) S = seed, IS = inner stem, OS = outer stem, WS = whole stem, L = leaf, R = root

| Family (No. proteins) | Enzyme Activity | Potential cell wall polymer | Reference | Organ |
|-----------------------|----------------|-----------------------------|-----------|-------|
| **Glycoside hydrolases** | | | | |
| GH1* (15) | β-glucosidase, | | 1,2 | All |
| GH3* (12) | β-xylanosidase, α-arabinofuranosidase | | 1,2 | All |
| GH5* (7) | β-mannanase | | 1,2 | All |
| GH9* (1) | Glucanase | Glucan, Hemicellulose (xyloglucan) | 1,2 | IS, OS, WS |
| GH10.3* (1) | Xylanase, Xylan endotransglycosylase | Hemicellulose (xylan) | 1,2,3 | S |
| GH16* (2) | Xyloglucan endotransglycosylase/hydrolase | Hemicellulose (xyloglucan) | 1,2 | OS, R |
| GH17 (5) | gluconase | MLG, Callose | 1,2 | WS, L, R, S |
| GH28 (4) | Polygalacturonase, pectinase | Pectin (homogalacturonan) | 1,2 | IS, WS |
| GH31* (4) | α-glucosidase, alpha-xylosidase | Hemicellulose (xyloglucan) | 1,2 | IS, OS, L, R, S |
| GH35 (6) | Galactosidase | Galactan | 1,2 | IS, OS, R, S |
| GH38 (4) | α-mannosidase | Glycoproteins | 2 | IS, OS, WS, R |
| GH51* (1) | α-arabinofuranosidase | Hemicellulose (xylan) | 1,2 | OS, WS |
| GH79.2 (1) | Glucuronidase | AGP | 4 | OS |
| GH95* (1) | α-fucosidase | Hemicellulose (xyloglucan), AGP | 2,4 | S |
| **Glycosyltransferases** | | | | |
| GT1 (12) | UDP-glycosyltransferase | Lignin (monolignols) | 5 | IS, S |
| GT2* (3) | Cellulose synthase, cellulose-synthase-like (mannan synthase, glucan synthase), glucan synthase) | Cellulose, hemicellulose: xyloglucan, (Gluco)mannan, MLG | 6,7 | IS |
| GT4 (7) | Sucrose Synthase | Cellulose | 6 | IS, OS, WS, R, S |
| GT8* (2) | Galacturonosyltransferase, glucuronosyltransferase; Galacturonosyltransferase, | Pectin (HG), hemicellulose: xylan | 7,8,9 | IS, OS, WS |
| GT47* (1) | Arabinosyltransferase, Xylosyltransferase, | Hemicellulose: Pectin (RGII), xyloglucan, xylan | 7,8,9 | IS, OS |
| GT48 (1) | Glucan synthase | Callose | 8 | S |
| GT75* (4) | Glucuronosyltransferase | Hemicellulose (xylan) | 10 | WS, S |
| **Auxilliary activity** | | | | |
| AA1 (11) | Phenoloxidase/Laccase | Lignin | 11 | IS, OS, S |
| AA2 (40) | Phenoloxidase/Peroxidase | Lignin | | All |
| **Carbohydrate esterases and Polysaccharide lyases** | | | | |
| CE8 (8) | Pectin methylesterase | Pectin | 2 | OS, WS, L, R, S |
| CE13 (2) | Pectin acetylsterase | Pectin | 2 | OS, R |
| PL4_2 (1) | Pectate lyase | Pectin | | OS |
| **Carbohydrate Binding Modules and Expansin** | | | | |
| CBM22* (2) | Xylanase | Hemicellulose (xylan) | 12 | WS |
| CBM43 (2) | Glucanase | MLG, Callose | 13 | WS, S |
| EXPN (2) | No enzyme activity | Cellulose-hemicellulose network | 14 | WS, L, S |
an important paralogy in comparison with the other species used for the analysis. The first (supplemental Fig. S8) corresponded to the GH19 family containing chitinase-like (CTL) proteins as previously reported (3, 6, 53). The second paralogy concerned the xyloglucan endo-transglycosylase/hydrolase family GH16 IIIA (39) that is associated with hemicellulose metabolism and cell wall expansion in plants (54) (Fig. 6, supplemental Fig. S9). Our results indicated the presence of 10 flax proteins in the class IIIA compared with 3 proteins in Arabidopsis and *Populus trichocarpa*. The flax XTH sequences group together and show bootstrap values suggesting a strong paralogy that might be related to cell wall specificities in this species. Overall, the flax genome contained a greater number of XTH genes (67) compared with poplar (44) and Arabidopsis (3, 33, 55).

We then determined the distribution of different hemicellulose types in our flax stems by immunochemistry (Fig. 7). As previously demonstrated (56, 8) cell walls in flax xylem tissue were extensively labeled by xylan LM10 and LM11 antibodies (Fig. 7 A, C). Although no labeling was observed in bast fiber cell walls with LM10 antibodies (Fig. 7 B), a relatively intense labeling was present in the middle lamella/primary cell wall of bast fibers with LM11 antibodies that recognize more heavily substituted xylans. The middle lamella/primary cell wall of bast fibers was heavily labeled by the xyloglucan antibodies LM15 and LM24 that recognize the XXXG and XXLG/XLXG oligosaccharide epitopes, respectively (Fig. 7 D, E). LM15 antibodies also recognized epidermal cell walls and gave a weak labeling in xylem walls (Fig. 7 D). The LM24 antibodies weakly labeled xylem and pith cell walls (Fig. 7 E). Finally, the LM21 antibody that recognizes mannan/heteromannans gave a strong signal in bast fiber secondary (but not primary) cell walls, as well as in the first few cell layers of the most recently formed xylem tissue as previously observed (8) (Fig. 7 F). Overall these results indicate that flax fiber cell walls

![Fig. 3. Repartition of the 465 flax Total CWPs identified in 4 different organs (A) and stem tissues (B). Whole stem (WS), Seeds (S), leaves (L), roots (R), Inner stem (IS), Outer stem (OS).]
Further information on the structure of flax hemicelluloses in inner- and outer-stem tissues plants was obtained by enzymatic fingerprinting. Analyses using MALDI-TOF-MS (Fig. 9) of a hemicellulose-rich fraction from inner stem tissues digested with endo(1→4)-β-D-xylanase (Fig. 9A) revealed 2 main known ions with m/z values of 759 and 781 corresponding to the (M+Na)⁺ and (M+2Na-H)⁺ adducts, respectively, of an oligosaccharide with a 4-O-MeGlcA residue linked to 4 pentose residues. Considering the monosaccharide composition of the fraction (supplemental Fig. S11), this fragment is most likely derived from the enzymatic degradation of a glucuronoxylan. Analyses of the same sample digested by endo-glucanase (Fig. 9B) allowed the identification of 4 ions at m/z values corresponding to different xyloglucan oligosaccharides (1085: XXXG; 1247: XLXG/XXLG, 1393: XXFG, 1555: XLF). These fragments are derived by enzymatic cleavage of nonsubstituted glucose residues of a fucosylated XXXG-type xyloglucan. Ions corresponding to the previously identified glucuronoxylan oligosaccharides (759, 781) as well as a new ion at m/z = 891 most likely indicative of a 4-O-MeGlcA residue linked to 5 pentose (xylose) residues were also present. The presence of xylan-derived oligosaccharides in the endo-glucanase-digested fraction would seem to suggest that the enzyme used is contaminated by xylanase activity as previously observed (57). MALDI-TOF-MS analyses of the hemicellulose-rich fraction from outer stem tissues digested with endo(1→4)-β-D-xylanase (Fig. 9C) revealed the presence of a major peak with m/z = 759 corresponding to the (M+Na)⁺ adduct of a 4-O-MeGlcA residue linked to 4 pentose (xylose) residues. Analyses of endo-glucanase-digested samples (Fig. 9D) allowed the identification of the same 4 peaks identified in inner-stem samples, but at much higher levels indicating that outer-tissue hemicelluloses are richer in xyloglucans. Two smaller peaks with m/z = 791 and 953 also revealed the presence of XXG and XXGG/GXXG xyloglucan oligosaccharides. No peaks corresponding to xylan oligosaccharides could be detected. Overall these results indicate that the hemicelluloses present in flax stem inner tissues are mainly xylans together with lesser amounts of xyloglucans. In contrast, outer stem tissue hemicelluloses are mainly xyloglucans.

**DISCUSSION**

**Creation of a Flax Cell Wall Proteome**—Flax is an economically important fiber species and represents an interesting model for studies in plant cell wall biology. Because the industrial quality of fibers is related to the structure of the cell wall, a better understanding of the metabolism underlying the formation and modifications of cell walls in this species should contribute to quality improvement. Although genomics, transcriptomics, and functional approaches have contributed to our knowledge about cell wall biology in flax, a detailed analysis of the flax cell wall proteome is currently lacking. As a first step toward completing this gap in our knowledge we used nanoLC-MS/MS to identify proteins pres-
ent in fractions enriched in soluble proteins, cell wall proteins and membrane proteins obtained from 4 different flax organs/tissues. The decision to use extraction protocols aimed at producing fractions enriched in proteins from different cellular compartments is justified because the term “cell wall protein” can be ambiguous. One interpretation requires that the protein is physically located within the cell wall and is based on the selection of proteins having a predicted signal peptide but no intracellular retention signal (47). However, many proteins that are not physically located in the cell wall are involved in the biosynthesis of major cell wall polymers and it is therefore of biological interest to also include these proteins in any overall study of cell wall metabolism. For example, most of lignin biosynthesis enzymes are cytosolic whereas different glycosyltransferases can be physically associated with the plasma membrane (cellulose synthesis) and/or the endoplasmic reticulum/Golgi apparatus (hemicellulose, pectin and glycoprotein synthesis). In our case the use of specific extraction protocols (58, 13) allowed us to obtain 82 additional cell wall related proteins.

Subsequent analyses of our data combined with publically available data (12, 13) allowed us to generate a flax cell wall proteome containing 465 nonredundant proteins of which 131 have not been previously identified. This data set was obtained by combining the results of 4 different expert curation analyses (cell wall related CAZy proteins, lignin biosynthesis enzymes, secreted proteins and other Mapman cell wall bin proteins) aimed at identifying all “cell wall-related proteins,” not just those proteins physically located in the cell wall and/or identified in the Mapman cell wall bin. The 465 cell-wall-related proteins obtained by this multi-level analysis compares favorably with another recent study on the maize leaf proteome (60) that used a more powerful three-phase capillary chromatography-MS/MS enabling the authors to identify low abundance proteins. Although the total number of proteins identified in maize (12,000) was much higher than in our study, the final maize leaf ‘cell wall proteome’ based on the single Mapman classification was lower (227 proteins).

**Flax Cell Wall Proteins Involved in Wall Metabolism and Defense**—An analysis of the number of identified proteins and enzyme families in different organs/tissues indicated that proteins associated with the biosynthesis of the major cell wall polymers were present in all organs except for leaves. Although it is likely that a large part of the observed differences can be related to the difficulty of obtaining low abundance proteins and/or extracting proteins from contrasted tissue types, it is reasonable to assume that some differences can be related to cell wall metabolism. The absence of proteins involved in both polysaccharide polymer- and lignin-biosynthesis in leaves is most likely related to the fact that flax leaves are small (~2 cm long) and were fully formed when sampled. At this stage, major polymer biosynthesis is completed and it is therefore not particularly surprising that no biosynthetic enzymes were identified in this organ. Glycosyltransferase 8 family proteins, potentially involved in pectin (homogalacturonan) and hemicellulose (xylan) biosynthesis were present in all the other organs/tissues except for seeds where only the GT75 family involved in xylan biosynthesis was represented. Pectin and hemicellulose biosynthesis were further represented in stem tissues by GT47 (Rhamnogalacturonan I, xyloglucan, xylan) and GT77 (Rhamnogalacturonan II/AGP) most likely reflecting the higher amount of cell wall biosynthesis occurring in this organ. In agreement with this conclusion was the observation that the GT2 family (cellulose synthase) was found in the inner-stem tissues characterized by secondary xylem formation and extensive secondary cell wall
A closer examination of the GT2 proteins showed that they are orthologs of 3 different Arabidopsis cellulose synthase enzymes (CesA3, CesA4 and CesA8), two of which are involved in secondary cell wall formation (61). The presence of these proteins is also in agreement with previous flax transcriptomics data (33, 9).

Xylem secondary cell wall formation also involves extensive lignin biosynthesis and deposition necessary for the mechanical reinforcement of this vascular tissue during sap transport. The presence of proteins representing 8 of the 11 canonical lignin biosynthetic families in flax inner- and whole-stem samples is therefore logical and supports previous transcriptomics data showing that monolignol biosynthesis enzymes are significantly more highly expressed in inner-stem tissues (20, 33, 4). Nevertheless, 3 lignin families were also represented in outer-stem tissues and may be related to the formation of...
Lignin phenylpropanoids responsible for UV protection, as well as limited lignification in these tissues (20, 62). Monolignol biosynthetic enzymes were also present in the seed cell wall proteome where they are probably related to the biosynthesis of lignans, biologically active dimers of monolignols (2, 63).

Plant cell walls undergo extensive remodeling during cell growth and many enzyme families potentially acting on pectins, hemicelluloses and glycoproteins were represented in the flax cell wall proteome. Globally, hemicellulose metabolism was the most represented class in all organs/tissues (including leaves) with enzyme families potentially acting on both xyloglucans (GH1, GH9, GH16, GH31, and GH95) and xylans (GH3, GH10, GH51, and CBM22) being present. Pectin remodeling activities were also represented in all organs/tissues. The observation that proteins associated with pectin and hemicellulose remodeling are more highly represented in outer-stem tissues when compared with inner-stem tissues, even though the latter tissue is characterized by extensive xylem-related cell wall formation is interesting. This could be related to the presence in outer tissues of bast fibers with thick secondary cell walls that undergo extensive noncellulosic polysaccharide (NCP) remodeling and restructuring during their maturation (5, 19, 23).

Cell growth and cell wall structure are not only determined by polymer biosynthesis and remodeling enzymes but are also dependent on several other proteins. Such proteins are particularly well represented in stem samples where orthologs of 5 different Fascilin-like arabinogalactan proteins are present, including 3 orthologs of the FLA12 protein functionally associated with stem tensile strength and stiffness in Arabidopsis and Eucalyptus (64). Similarly, expansins acting on the cellulose-hemicellulose network during cell growth are also only found in whole stem samples (65). Several proteases potentially associated with developmental programmed cell death were also present in stems, roots, and seeds (66).

Cell walls are also an important interface between the plant and its environment, and cell wall proteins therefore play a nonnegligible role in responses toward abiotic stress and pathogens (biotic stress). Analysis of cell wall proteins involved in abiotic stress responses indicated that proteins belonging to this class were abundant in whole stem and seed samples, followed by leaves and roots. When inner- and outer-stem tissues were compared, proteins were more highly abundant in the latter. Highly represented proteins in this class included peroxidases, potentially associated with elimination of stress-induced ROS and/or promoting programmed cell death (PCD) as part of the overall stress response (67, 68). Proteins involved in managing protein mis-folding under stress conditions were also present and included calreticulins (CRT) and protein disulfide isomerase-like (PDIL) proteins, both of which play a central role in Endoplasmic Reticulum (ER) quality control under stress conditions (69, 70). Another well-represented group included aspartyl proteases that are also involved in the regulation of PCD during development and stress (71). Other proteins previously associated with stress responses in plants and present in the flax abiotic stress block included cupins, GLPs and osmotin (18, 72, 73).

The defense (biotic stress) class of cell wall proteins is also well represented in whole stems (more so in outer tissues compared with inner tissues) and seeds, but less so in leaves and roots. Identified proteins include those potentially involved in pathogen detection (LRR type proteins/receptors), signalization (lipase/lipoxygenase) and responses (Germin-like proteins, chitinases, serine carboxypeptidases, and trypsin/protease inhibitors (74–78).

**Fig. 7. Immunolocalization of flax stem hemicellulose epitopes.** Pre-immune serum (A); anti-xylan LM10 antibodies (B); anti-xylan arabinxyylan LM11 antibodies (C); anti-xyloglucan LM15 antibodies (D); anti-xyloglucan LM24 antibodies (E); anti-mannan/heteromannan LM21 antibodies (F). cz: cambial zone; ep: epidermis; f: fiber; x: xylem; r: xylem ray; v: xylem vessel/tracheid. Bar = 100 μm (except for zoom).
Flax Cell Wall Proteome

**Fig. 8.** Middle infra-red spectra at 1800–900 cm$^{-1}$ of bast fiber cell walls (A) and secondary derivative (B) showing significant peaks characteristic of hemicelluloses (C).
Flax Cell Wall Proteome

Flax Stem Hemicelluloses and XTH Paralogy—Flax bast fibers are characterized by an extremely thick cell wall that is made up of a thin primary cell wall and secondary S1 layer, together with a much thicker cellulose-rich S2 layer that is also referred to as a gelatinous layer (G-layer) as it presents many similarities with the same layer in tension wood G fibers (19, 20, 23). Although it is the cellulose microfibrils that provide the main load-bearing structure in the flax fiber cell wall and are responsible for its remarkable mechanical properties, it is likely that other noncellulosic polysaccharides (NCPs) also contribute to the overall performance of the flax fiber. Possible candidates include beta-1,4 galactans that are progressively degraded by a GH35 family beta galactosidase resulting in a closer association between cellulose microfibrils in the matur- ing S2 (G-layer) (5). Interestingly, xyloglucans appear to play a similar role in the S2 (G-layer) of tension wood gelatinous fibers (23). The overall hemicellulose content of flax fibers varies from 4.7% (unretted fibers) to 8.9% (retted fibers) and contains both xylans and xyloglucans in agreement with our identification of proteins potentially involved in the biosynthesis and remodeling of these two types of polymer (49, 50). Our chemical, immunological and spectroscopic results confirmed the presence of xyloglucans, as well as of some substituted xylans in flax outer tissues/fibers. In contrast to the beta-1,4 galactan that is mainly localized in the S2/G-layer of flax fibers the xyloglucans (and xylans) were localized to the primary cell wall and S1 layers. Labeling by LM21 antibody also indicated the presence of beta-1,4 mannans (glucomannans/galactomannans) mainly in the older parts of the S2/G-layer, as well as more weakly in the S1 layer. Fingerprinting analyses of hemicelluloses from outer-stem tissues indicated that the presence of a fucosylated XXXG-type xyloglucan. This contrasts with similar analyses of hemicellulose fractions from linseeds where XLLG was shown to be the most represented oligo-xyloglucan and underlines organ-/tissue-specificities (79). Overall the observed population of flax oligo- xyloglucons is like that of Arabidopsis suggesting that the xyloglucans in the flax fiber primary- and S1 secondary-cell wall are similar to those in other Eudicots despite the particular structure of the fiber S2/G-layers (80). The flax xylan oligosaccharide fragment composition released by an endo- xylanase was similar in seeds, and inner- and outer-stem tissues are very similar with a xylose tetrasaccharide fragment carrying one 4-O-MeGlcA residue being the most abundant in all 3 tissues. This fragment is derived from the endoxylanase hydrolysis of a glucuronoxylan. Once again, this pattern of substitution is similar to that observed in Arabidopsis (81). Overall these results confirm the presence of fucosylated xyloglucans, and to a more limited extent glucuronoxylans, in flax fiber cell walls.

As well as being characterized by thick, cellulose-rich cell walls, flax fibers are also extremely long cells and can reach lengths of up to 70 mm. Their development therefore requires careful coordination between cell loosening mechanisms allowing rapid cell expansion and cell wall polymer biosynthesis necessary for secondary cell wall formation (23, 82). A major player in growth-related cell wall remodeling is the xyloglucan endo-transglycosylase/hydrolase (XTH) enzyme that cuts (hydrolase activity) xyloglucan hemicellulose polymers linking cellulose microfibrils and is believed to favor cell expansion during cell growth although recent work has cast doubt upon this generally accepted idea (52, 83, 84, 85). Subsequently, the XTH relinks (endotransglycosylation activity) the cut end of the xyloglucan to another xyloglucan polymer thereby relinking cellulose microfibrils and contributing to stopping cell expansion (83, 54). Both XTH activity and gene expression have previously been correlated with G-fiber formation in tension wood in poplar and, given i) the presence of xyloglucons in the flax fiber cell wall and ii) previous transcriptomics data, it is not surprising to find an XTH protein in fiber-containing tissues in flax (4, 23, 33, 54, 86, 87, 88, 89). Analyses showed that the flax XTH gene family contains 61 predicted genes and is much larger than in other species such as Arabidopsis (33), poplar (41) and tomato (3, 25, 55). Based on enzymatic and phylogenetic studies plant XTHs are divided into 2 main groups, the class I/II and the class IIIA (83). Class I/II XTHs show xyloglucan endotransglycosylase activity (XET; EC 2.4.1.207) and are classically believed to be involved in cutting/rejoining xyloglucans during cell extension processes (90, 55). In contrast class IIIA XTHs show xyloglucan endo-hydrolase activity (XEH; EC 3.2.1.115) and are involved in cutting/degrading xyloglucans, but show no/little transglycosylation activity. The class IIIA includes the archetypal nasturtium (Tropaeolum majus) xyloglucanase1 TmNXG1 that hydrolyzes seed storage xyloglucan during germination (91). The flax XTH protein identified in our studies belongs to the class IIIA and is therefore likely to show xyloglucan endo-hydrolase activity rather than a transglycosylation activity. Interestingly, phylogenetic analyses of the class IIIA family in flax revealed an important paralogy with 10 flax class IIIA XTHs as compared with 3 in both Arabidopsis and poplar. Although flax has undergone 2 whole genome duplications during its evolution, it is unlikely that the observed XTH paralogy is related to these whole genome events because we did not observe comparable duplications in the other cell wall families apart from that previously noted for the

Fig. 9. MALDI-TOF-MS spectra of fragment ions (M+Na)+ corresponding to oligosaccharides released by enzymatic digestion of 2-month-old flax stems. Inner stem tissue sample digested by endoxylanase (A) or endo-beta-glucanase (B), outer stem tissue sample digested by endoxylanase (C) or endo-beta-glucanase (D). Figs. refer to m/z values of xylan-derived fragments (759: X-4OMeGlcA + Na+, 781: X-40MeGlcA + 2Na+ - H+, 891: X-40MeGlcA + Na+) and xyloglucan-derived fragments (1085: XXXG + Na+, 1247: XLXG/XXLG + Na+, 1393: XXFG + Na+, 1595: XLFG + Na+).
GH19 family containing chitinase-like (CTL) genes (3, 6, 53). Although further analyses are necessary the large number of CTL and XTH paralogs might suggest that the expansion in these 2 cell-wall related gene families is related to the particular cell wall architecture of flax fibers. In support of this argument is the observation that CTL genes have been associated with the formation of cellulose-rich G-type cell walls in other species and specific flax CTL genes are more highly expressed in flax fiber-bearing tissues (6). Furthermore, a comparative genomic approach of xyloglucan-related genes in green plants showed that although no XTH genes are found in chlorophyte green algae, 2 XTH founder genes are present in charaophytes, one of which has undergone rapid expansion in embryophytes (92). Such an observation would suggest that the acquisition of xyloglucan, and xyloglucan remodeling capacity have played an important role in land colonization and the capacity of embryophytes to occupy different niches (92, 93).

XTH activity in aspen tension wood fibers is proposed to be necessary for both cell elongation and for maintaining/repairing xyloglucan links between the S-layers and G-layer thereby contributing to fiber cell wall structural integrity (23, 94). Our results identifying xylangs, as well as ferulic acid residues in the flax fiber cell wall could also suggest that xylan hemicelluloses contribute to cell wall integrity in these cells. In addition, has also been suggested that XTHs contribute to trimming xyloglucans attached to newly formed cellulose microfibrils facilitating the assembly of cellulose microfibrils (95). However, unlike tension wood fibers, the major NCP of the S2/G-layer in flax is not xyloglucan, but a beta-1,4 galactan (23). Although such an observation could suggest that the flax XTHs are more likely involved in the cell elongation process it is important to keep in mind recent studies casting doubt upon the role of xyloglucans in cell expansion (84, 85). Alternatively, it is possible that the flax XTH is part of a xyloglucan recycling pathway as has been suggested in arabidopsis (96, 90). Overall these results highlight the likely important role of xyloglucans and XTHs in flax fiber cell wall metabolism. The availability of flax mutant populations and the recent development of a flax VIGS system open the door to future functional studies that should allow us to get a better understanding of these processes (7, 9).

REFERENCES

1. Singh, K. K., Mridula, D., Rehal, J., and Barnwal, P. (2011) Flaxseed: A potential source of food, feed and fiber. Crit. Rev. Food Sci. Nutr. 51, 210–222

2. Touré, A., and Xueming, X. (2010) Flaxseed lignans: Source, biosynthesis, metabolism, antioxidant activity. Bio-active components, and health benefits. Compr. Rev. Food Sci. Food Saf. 9, 261–269

3. Wang, Z., Hobson, N., Galindo, L., Zhu, S., Shi, D., McDill, J., Yang, L., Hawkins, S., Neutelings, G., Datia, R., Lambert, G., Galbraith, D. W., Grassa, C. J., Geraldes, A., Cronk, Q. C., Cullis, C., Dash, P. K., Kumar, P. A., Cloutier, S., Sharpe, A. G., Wong, G. K.–S., Wang, J., and Deyhols, M. K. (2012) The genome of flax (Linum usitatissimum) assembled de novo from short shotgun sequence reads. Plant J. 72, 461–473

4. Huis, R., Moree, K., Finiaux, O., Lucau-Danilla, A., Fenart, S., Grec, S., Neutelings, G., Chabbert, B., Mesnard, F., Boerjan, W., and Hawkins, S. (2012) Natural hypolignification is associated with extensive oligogalacturonol accumulation in flax stems. Plant Physiol. 158, 1893–1915

5. Roach, M. J., Mokshina, N. Y., Badhan, A., Snegireva, A. V., Hobson, N., Deyhols, M. K., and Gorshkova, T. A. (2011) Development of cellulosic secondary walls in flax fibers requires –galactosidase. Plant Physiol. 156, 1351–1363

6. Mokshina, N., Gorshkova, T., and Deyhols, M. K. (2014) Chitinase-like (CTL) and cellulose synthase (CESA) gene expression in gelatinous-type cellulosic walls of flax (Linum usitatissimum L.) bast fibers. PLoS ONE 9, e97949

7. Chantreau, M., Grec, S., Gutierrez, L., Dalmais, M., Pineau, C., Demailly, H., Paysant-Leroux, C., Tavernier, R., Trouvé, J.–P., Chatterjee, M., Guillot, X., Brunaud, W., Chabbert, B., Van Wuytswinkel, O., Bendahmane, A., Thomasset, B., and Hawkins, S. (2013) PT-Flax (phenotyping and TILLing of flax): development of a flax (Linum usitatissimum L.) mutant population and TILLing platform for forward and reverse genetics. BMC Plant Biol. 13

8. Chantreau, M., Portelette, A., Dauve, R., Kyoto, S., Crönier, D., Moree, K., Arribat, S., Neutelings, G., Chabi, M., Boerjan, W., Yoshinaga, A., Mesnard, F., Grec, S., Chabbert, B., and Hawkins, S. (2014) Ectopic lignification in the flax lignified bast fiber mutant stem is associated with tissue-specific modifications in gene expression and cell wall composition. Plant Cell Online 26, 4462–4482

9. Chantreau, M., Chabbert, B., Billiard, S., Hawkins, S., and Neutelings, G. (2015) Functional analyses of cellulose synthase genes in flax (Linum usitatissimum) by virus-induced gene silencing. Plant Biotechnol. J. 13, 1312–1324

10. Ibragimová, N. N., Mokshina, N. E., and Gorshkova, T. A. (2012) Cell wall proteins of flax phloem fibers. Russ. J. Bioorganic Chem. 38, 117–125

11. Hotte, N. S. C., and Deyhols, M. K. (2008) A flax fibre proteome: identification of proteins enriched in bast fibres. BMC Plant Biol. 8, 52

12. Barvkar, V. T., Pardeshi, V. C., Kale, S. M., Kadoo, N. Y., Giri, A. P., and Gupta, V. S. (2012) Proteome profiling of flax (Linum usitatissimum) seed: Characterization of functional metabolic pathways operating during seed development. J. Proteome Res. 11, 6264–6276

13. Day, A., Fénart, S., Neutelings, G., Hawkins, S., Rolando, C., and Tokarski, A. (2013) Identification of cell wall proteins in the flax (Linum usitatissimum) stem. Proteomics 13, 812–825

14. Haider, S., and Pal, R. (2013) Integrated analysis of transcriptomic and proteomic data. Curr. Genomics 14, 91–110

15. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. 19, 1720–1730

16. Rossignol, M., Pettier, J. B., Mock, H. P., Matros, A., Maldonado, A. M., and Jorrin, J. V. (2006) Plant proteome analysis: A 2004–2006 update. Proteomics 6, 5529–5548

17. Cui, S., Hu, J., Guo, S., Wang, J., Cheng, Y., Dang, X., Wu, L., and He, Y. (2012) Proteome analysis of Physcomitrella patens exposed to progressive dehydration and rehydration. J. Exp. Bot. 63, 711–726

18. Gábríšová, D., Klubícová, K., Danchenko, M., Goemöry, D., Berezhna, V. V., GaBrísova, D., Klubicová, K., Danchenko, M., Goemöry, D., Berezhna, V. V., TILLing of flax): development of a flax (Linum usitatissimum) mutant stem requires -galactosidase. Plant Cell Physiol. 49, e97949

19. Morvan, C., Andème-Onzighi, C., Girault, R., Himmelsbach, D. S., Driouich, A., and Akin, D. E. (2003) Building flax fibres: More than one brick in the wall. Plant Physiol. Biochem. 41, 935–944

20. Day, A., Ruel, K., Neutelings, G., Crönier, D., David, H., Hawkins, S., and Chabbert, B. (2005) Lignification in the flax stem: Evidence for an unusual lignin in bast fibers. Planta 222, 234–245
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21. van Hazendonk, J. M., Reinerik, E. J. M., de Waard, P., and van Dam, J. E. G. (1996) Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (Linum usitatissimum L). Carbohydr. Res. 291, 141–154

22. Style, C. (2002) Analysis of the flax fibres tensile behaviour and analysis of the tensile stiffness increase. Compos. - Part A Appl. Sci. Manuf. 33, 939–946

23. Mellerowicz, E. J., and Gorshkova, T. A. (2012) Tensional stress generation in gelatinous fibres: A review and possible mechanism based on cell-wall structure and composition. J. Exp. Bot. 63, 551–565

24. Davis, E. A., Derouet, C., Herve Du Penhoat, C., and Morvan, C. (1998) Isolation and an N.M.R. study of pectins from flax (Linum usitatissimum L.). Carbohydr. Res. 197, 205–215

25. Girault, R., Bert, F., Rhoayou, C., Jauneau, A., Morvan, C., and Jarvis, M. (1997) Galactans and cellulose in flax fibres: Putative contributions to the tensile strength. Int. J. Biol. Macromol. 21, 179–188

26. Gorshkova, T., and Morvan, C. (2006) Secondary cell-wall assembly in flax phloem fibres: Role of galactans. Plantas 223, 149–158

27. Boerjan, W., Ralph, J., and Baucher, M. (2003) Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546

28. McFarlane, H. E., Döring, A., and Persson, S. (2014) The cell biology of cellulose synthesis. Annu. Rev. Plant Biol. 65, 69–94

29. Scheller, H. V., and Ulvskov, P. (2010) Hemicelluloses. Annu. Rev. Plant Biol. 61, 263–289

30. Atmodjo, M. a., Hao, Z., and Mohnen, D. (2013) Evolving views of pectin biosynthesis. Annu. Rev. Plant Biol. 64, 747–779

31. Franková, L., and Fry, S. C. (2013) Biochemistry and physiological roles of enzymes that “cut and paste” plant cell-wall polysaccharides. J. Exp. Bot. 64, 3519–3550

32. Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497

33. Feiz, L., Irshad, M., Pont-Lezica, R. F., Canut, H., and Jamet, E. (2006) A genome-wide phylogenetic reconstruction of family 1 UDP-glycosyltransferases revealed the expansion of the family during the adaptation of plants to life on land. Plant J. 48, 1030–1042

34. Manabe, Y., Verheertbruggen, Y., Gille, S., Harholt, J., Chong, S.-L., Pawar, P. M., Mellerowicz, E. J., Tenkanen, M., Cheng, K., Pauly, M., and Scheller, H. V. (2013) Reduced Wall Acetylation proteins play vital and distinct roles in cell wall O-acetylation in Arabidopsis. Plant Physiol. 163, 1107–1117

35. Sen Clemente, H., and Jamet, E. (2015) WallProtDB, a database resource for flax cell wall proteomes. Plant Mol. Biol. 80, 111–120

36. Roudier, F., Fernandez, A. G., Fujita, M., Himmelsbach, R., Bommer, G. H. H., Schindelman, G., Song, S., Baskin, T. I., Dupree, P., Wasteneys, G. O., and Beney, P. N. (2005) COBRA, an Arabidopsis extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. Plant Cell 17, 1749–1763

37. McLaughlin, G. J. (1993) Isolation and partial characterisation of the non-cellulosic polysaccharides of flax fibre. Carbohydr. Res. 241, 227–236

38. Sharma, H. S. S., Faughey, G., and Lyons, G. (1999) Comparison of physical, chemical, and thermal characteristics of water-, and, 139–143

39. Carpita, N. C., and Mccann, M. C. (2000) in Biochemistry and Molecular Biology of Plants, pp. 52–109

40. Cosgrove, D. J. (2005) Growth of the plant cell wall. Nat. Rev. Mol. Cell Biol. 6, 850–861

41. Svinhinnson, S., Mccill, J., Wong, G. K. S., Li, J., Li, X., Deyholos, M. K., and Cronk, C. Q. B. (2014) Phylogenetic pinpointing of a paleopolyploidy event within the flax genus (Linum) using transcriptomics. Ann. Bot. 113, 753–761

42. Nishikubo, N., Takahashi, J., Roos, A. A., Derba-Macelu, M., Piens, K., Brummer, H., Teeri, T. T., Stålbbrand, H., and Mellerowicz, E. J. (2011) Xyloglucan endo-transglycosylase-mediated xyloglucan rearrangements in developing wood of hybrid aspen. Plant Physiol. 155, 399–413

43. Shi, Y. Z., Zhu, X. F., Miller, J. G., Gregson, T., Zheng, S. J., and Fry, S. C. (2015) Distinct catalytic capacities of two aluminium-repressed Arabidopsis thaliana xyloglucan endotransglycosylase/hydrolases, XTH15 and XTH31, heterologously produced in Pichia. Phytochemistry 112, 165–170

44. McCartney, L., Marcus, S. E., and Knox, J. P. (2005) Monoclonal antibodies to plant cell wall xylans and arabinoxylans. J. Biol. Chem. 280, 2802–2806

45. Cosgrove, D. J. (2005) Growth of the plant cell wall. Nat. Rev. Mol. Cell Biol. 6, 543–546

46. Thomson, J. A. (1993) Molecular biology of xylan degradation. FEMS Microbiol. Lett. 104, 65–82

47. Song, D., Xi, W., Shen, J., Bi, T., and Li, L. (2011) Characterization of the plasma membrane proteins and receptor-like kinases associated with secondary vascular differentiation in poplar. Plant Mol. Biol. 76, 97–115

48. Day, A., Fénart, S., Neutelings, G., Hawkins, S., Rolando, C., and Tokarski, C. (2013) Identification of cell wall proteins in the flax (Linum usitatissimum) stem. Proteomics 13, 812–825

49. Facette, M. R., Shen, Z., Björnsdottrir, F. R., Briggs, S. P., and Smith, L. G. (2013) Parallel proteomic and phosphoproteomic analyses of successive stages of maize leaf development. Plant Cell 25, 2798–2812

50. Fernández-Blanco, C., Feng, D. X., Hu, J., Sánchez-Vallet, A., Deslandes, L., Llorente, F., Berrocal-Lobo, M., Keller, H., Barlet, X., Sánchez-Rodríguez, C., Anderson, L. K., Somerville, S., Marco, Y., and Molina, A. (2007) Impairment of cellulose synthases required for Arabidopsis secondary cell wall formation enhances disease resistance. Plant Cell 19, 890–903

51. Dixon, R. A., and Paiva, N. L. (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097

52. Venglat, P., Xiang, D., Qiu, S., Stone, S. L., Tibiche, C., Cram, D., Alting-Mees, M., Nowak, J., Cloutier, S., Deyholos, M., Bekkaoui, F., Sharpe, A., Wang, E., Rowland, G., Selvaraj, G., and Datla, R. (2011) Gene expression analysis of flax seed development. BMC Plant Biol. 11, 74

53. MacMillan, C. P., Mansfield, S. D., Stachurski, Z. H., Evans, R., and Southerton, S. G. (2010) Fasciclin-like arabinogalactan proteins: Specialization
for stem biomechanics and cell wall architecture in Arabidopsis and Eucalyptus. Plant J. 62, 689–703
65. McQueen-Mason, S. J., and Cosgrove, D. J. (1995) Expansion mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding. Plant Physiol. 107, 87–100
66. Sueldo, D. J., and van der Hoorn, R. A. L. (2017) Plant life needs cell death, but does plant cell death need Cys proteases? FEBS J. 1–9
67. Choudhury, F. K., Rivero, R. M., Eduardo Blumwald, and Mittler, R. (2016) Reactive oxygen species, abiotic stress and stress combination. Plant J. 1–12
68. Mittler, R. (2016) ROS Are Good. Trends Plant Sci. 9
69. Kim, Y. J., Yeu, S. Y., Park, B. S., Koh, H.-J., Song, J. T., and Seo, H. S. (2012) Protein Disulfide Isomerase-Like Protein 1–1 Controls Endosperm Development through Regulation of the Amount and Composition of Seed Proteins in Rice. PLoS ONE 7, e44493
70. Silva, P. A., Silva, J. C. F., Caetano, H. D., Machado, J. P. B., Mendes, G. C., Reis, P. A., Brustolino, O. J., Dal-Bianco, M., and Fontes, E. P. (2015) Comprehensive analysis of the endoplasmic reticulum stress response in the soybean genome: conserved and plant-specific features. BMC Genomics 16, 783
71. Kabbage, M., Kessens, R., LC, B., and, B., W. (2017) The life and death of a plant cell. Annu. Rev. Plant Biol. 68
72. Davidson, R. M., Reeves, P. A., Manosalva, P. M., and Leach, J. E. (2009) Germins: A diverse protein family important for crop improvement. Plant Sci. 177, 499–510
73. Anil Kumar, S., Hima Kumari, P., Shravan Kumar, G., Mohanalatha, C., and Kavi Kishor, P. B. (2015) Osmotin: a plant sentinel and a possible agonist of mammalian adipocytin. Front. Plant Sci. 6, 163
74. Jones, J. D. G., and Dangl, J. L. (2006) The plant immune system. Nature 444, 323–329
75. Feussner, I., and Wasternack, C. (2001) THE LIPOXYGENASE PATHWAY. SGM LaTeX2e Annu. Rev. Plant Biol. 5, 275–297
76. Collinge, D., Kragh, K., Mikkelsen, J., Nielsen, K., Rasmussen, U., and Vad, K. (1993) Plant chitinases. Plant J. 3, 31–40
77. Li, L., Xu, X., Chen, C., and Shen, Z. (2016) Genome-wide characterization and expression analysis of the Germin-like protein family in rice and arabidopsis. J. Int. Mol. Sci. 17
78. Ryan, C. a. (1990) Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. 28, 425–449
79. Ray, S., Paynel, F., Morvan, C., Lerouge, P., Drouich, A., and Ray, B. (2013) Characterization of mucilage polysaccharides, arabinogalactanproteins and cell-wall hemi-cellulosic polysaccharides isolated from flax seed meal: A wealth of structural moieties. Carbohydr. Polym. 93, 651–660
80. Obel, N., Erben, V., Schwarz, T., K??tnel, S., Fodor, A., and Pauly, M. (2009) Microanalysis of plant cell wall polysaccharides. Mol. Plant 2, 922–932
81. Rennie, E. A., and Scheller, H. V. (2014) Xylan biosynthesis. Curr. Opin. Biotechnol. 25, 100–107
82. Snegireva, a. V., Ageeva, M. V., Amenitskii, S. I., Chernova, T. E., Ebskamp, M., and Gorschkova, T. a. (2010) Intrusive growth of sclerenchyma fibers. Russ. J. Plant Physiol. 57, 342–355
83. Eklof, J. M., and Brumer, H. (2010) The XTH gene family: an update on enzyme structure, function, and phylogeny in xyloglucan remodeling. Plant Physiol. 153, 456–466
84. Park, Y. B., and Cosgrove, D. J. (2012) A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. Plant Physiol. 158, 1933–1943
85. Park, Y. B., and Cosgrove, D. J. (2015) Xyloglucan and its interactions with other components of the growing cell wall. Plant Cell Physiol. 56, 180–194
86. Nishikubo, N., Awano, T., Banasiak, A., Bourquin, V., Ibatullin, F., Funada, R., Brumer, H., Teeri, T. T., Hayashi, T., Sundberg, B., and Mellerowicz, E. J. (2007) Xyloglucan endo-transglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar - A glimpse into the mechanism of the balancing act of trees. Plant Cell Physiol. 48, 843–855
87. Baba, K., Park, Y. W., Kaku, T., Kaida, R., Takeuchi, M., Yoshida, M., Hosoo, Y., Ojio, Y., Okuyama, T., Taniguchi, T., Ohmiya, Y., Kondo, T., Shani, Z., Shoseyov, O., Awano, T., Serada, S., Norioka, N., Norioka, S., and Hayashi, T. (2009) Xyloglucan for generating tensile stress to bend tree stem. Mol. Plant 2, 893–903
88. Day, A., Addi, M., Kim, W., David, H., Bert, F., Mesnage, P., Rolande, C., Chabbert, B., Neutelings, G., and Hawkins, S. (2005) ESTs from the fibre-bearing stem tissues of flax (Linum usitatissimum L): Expression analyses of sequences related to cell wall development. Plant Biol. 7, 23–32
89. Roach, M. J., and Deyholos, M. K. (2007) Microarray analysis of flax (Linum usitatissimum L) stems identifies transcripts enriched in fibre-bearing phloem tissues. Mol. Genet. Genomics 278, 149–165
90. Kaewthai, N., Gendre, D., Eklof, J. M., Ibatullin, F. M., Ezcurra, I., Bhalerao, R. P., and Brumer, H. (2013) Group III-A XTH genes of Arabidopsis encode predominant xyloglucan endohydrolases that are dispensable for normal growth. Plant Physiol. 161, 440–454
91. Baumann, M. J., Eklof, J. M., Michel, G., Kallas, A. M., Teeri, T. T., Czjzek, M., and Brumer, H. (2007) Structural evidence for the evolution of xyloglucanase activity from xyloglucan endo-transglycosylases: biological implications for cell wall metabolism. Plant Cell 19, 1947–1963
92. Luiz, E. V., Bem, D., and Ga Vincenz, M. (2010) Evolution of xyloglucan-related genes in green plants. BMC Evol. Biol. 10, 341
93. Peña, M. J., Darvill, A. G., Eberhard, S., York, W. S., and O’Neill, M. A. (2008) Moss and liverwort xyloglucans contain galacturonic acid and are structurally distinct from the xyloglucans synthesized by hornworts and vascular plants. Glycobiology 18, 891–904
94. Gorshkova, T., Moskina, N., Chernova, T., Ibragimova, N., Sainkov, V., Mikshina, P., Tryfona, T., Banasiak, A., Immerzeel, P., Dupree, P., and Mellerowicz, E. J. (2015) Aspen tension wood fibers contain β-(1→4)-galactans and acidic arabinogalactans retained by cellulose microfibrils in gelatinous walls. Plant Physiol. 169, 2048–2063
95. Mellerowicz, E. J., and Sundberg, B. (2008) Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties. Curr. Opin. Plant Biol. 11, 293–300
96. Gun, M., Neumetzler, L., Kraemer, F., de Souza, a., Schultink, a., Pen, M., York, W. S., and Pauly, M. (2011) AXY8 encodes an -fucosidase, under-scoring the importance of apoplastic metabolism on the fine structure of Arabidopsis cell wall polysaccharides. Plant Cell 23, 4025–4040