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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 publically available data on flax seed and whole-stem proteins to generate a flax 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.

Immunolocalisation, FT-IR microspectroscopy, and enzymatic fingerprinting indicated that flax fiber primary/S1 cell walls contained xyloglucans with typical substituted side chains as well as glucuronoxylans in much lower quantities. These results suggest a likely central role of xyloglucans and endotransglucosylase/hydrolase activity in flax fiber formation and cell wall remodeling processes. *Molecular & Cellular Proteomics* 16: 10.1074/mcp.M116.063727, 1634–1651, 2017.

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-resinol 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-

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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, Predator, 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)¹ 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 cellulose-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 $\mu\text{mol.s/m}^2$, and hygrometry was 60%. 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 \times 3 sub-fractions \times 2 biological replicates. Two technical replicates were performed for each biological replicate. Only proteins identified in both biological replicates were retained for further analyses.

¹ The abbreviations used are: AA, Auxilliary activities; AGPs, Arabinogalactan proteins; AIR, Alcohol insoluble residue; ALA, Alpha linolenic acid; CAZy, Carbohydrate Active enZyme; CBM, Carbohydrate binding modules; CE, Carbohydrate esterases; CSC, Cellulose Synthase complex; CWP, Cell wall proteins; FT-IR, Fourier Transform Infrared; GH, Glycosyl hydrolases; G-layer/fibers, Gelatinous layer/fibers; GT, Glycosyl transferases; ID, Proteins with interacting domains; LM, Lipid metabolism; M, Miscellaneous; NCP, Noncellulosic polysaccharides; OR, Oxidoreductases; P, Proteases; PAC, Proteins acting on carbohydrates; PIC, Protease inhibitor cocktail; PL, Polysaccharide lyases; PVPP, Polyvinylpyrrolidone; S, Signaling; SDG, secoisolaricresinol diglucoside; SP, Structural proteins; UF, Proteins with unknown functions; UGT, UDP-glycosyltransferase; XEH, Xyloglucan endo-hydrolase; XET, Xyloglucan endotransglycosylase; XTH, Xyloglucan endo-transglycosylase/hydrolase.

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 protamine sulfate (1 mg/1 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 M 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 M 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 compatible (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 × 12 wells, Bio-Rad). The gel was stained with Instant Blue (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 enrichment 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.phytozome.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: Carbamidomethyl (C), dynamic modifications: Dioxidation (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 Phytozome (<http://www.phytozome.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.irsv.ups-tlse.fr/Wall-ProtDB/) 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 phytozome 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. *nagariensis*, *M. pusilla* CCMP1545, *O. lucimarinus*, *M. esculenta*, *A. coerulea*, *C. sinensis*, *E. grandis*, *G. raimondii*, *S. purpurea*, *V. vinifera*, *P. virgatum*). The number of taxa was limited to present an easily understandable 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 recuperation. This step was repeated 3 times and the pellet (=AIR) recovered and lyophilized (Fry, 1988). A hemicellulose fraction was obtained 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 containing flax hemicelluloses was dissolved in 2.5 ml ammonium acetate (10 mM, pH 5.5) and 0.5 ml digested by either (1) 2.5 units of endo-β-glucanase (Megazyme) for 18 h at 37 °C to generate oligo-xyloglucans or (2) 1 unit of endo-xylanase (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-xyloglucans/xylans) lyophilized and then dissolved in ultra-pure water.

MALDI-TOF-MS—One microliter of oligo-xyloglucans/xylans was cocrystallized with 1 μl 2,5 dihydrobenzoic acid prepared by dissolving 5 mg DHB in 500 μl acetonitrile/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 calibrated 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 dehydrated in an alcohol series and infiltrated with Technovit 7100 resin (Kulzer). Sections (3 μm) were cut using a Leica RM 2065 microtome and stained with a 0.5% (w/v) aqueous solution of Toluidine Blue O (TBO) (Sigma) for examining tissue organization. Sections were examined 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 immersion 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, xyloglucan epitopes with LM-15–24 and mannan/heteromannan 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 mM KH₂PO₄, pH 6.8, 2% (w/v) BSA, 0.01% (v/v) Triton X-100). After rinsing in three 10 min changes of PBS at

room temperature, sections were incubated with 1:100 diluted (PBS) goat antirat antibodies conjugated with FITC (fluorescein isothiocyanate, Jackson Immunoresearch Labs, West Grove, PA), for 1 h 30 at 37 °C and finally rinsed three times in PBS. Sections were mounted in Fluoromount™ (EMS, Fort Washington, PA) and conserved at 4 °C. Observations were done on a Leica microscope under ultraviolet irradiation, at λ_{exc} = 480 nm: λ_{em} = 505 nm for FITC λ_{exc} = 365 nm: λ_{em} = 420 nm for cellulose autofluorescence, or in bright field with phase contrast. In control sections, primary antibodies were replaced by preimmune rat serum or water. No signal was obtained on control sections.

FT-IR Microspectroscopy—Flax stem samples were dehydrated in a graded ethanol series (2 × 30 min, RT), followed by HistoClear (VWR, 2 × 1h), followed by Paraplast+ (Sigma) embedding. Ten micrometers thick transversal sections were obtained using a rotary microtome (Leica RM2065) and placed on CaF₂ windows (Crystran, U.K.) before being deparaffinized (HistoClear, 2 × 10 min, RT) rinsed in absolute ethanol (2 × 10 min, RT), air dried and stored under dust-free conditions.

FTIR spectra were acquired in transmission mode with a Bruker FTIR microscope Hyperion 3000 coupled to a Bruker Vertex 70 spectrometer equipped with a liquid nitrogen cooled MCT detector. Two hundred and fifty-six scans were recorded at 4 cm⁻¹ spectral resolution in the 650 cm⁻¹–4000 cm⁻¹ spectral range using a 36x Cassegrainian objective (NA = 0.5). The area of interest (10 μm × 50 μm) was selected by adjusting the knife-edge aperture so that only the area of interest is exposed for analysis. Only absorption band maxima in the 1800–900 cm⁻¹ spectral range were used to identify cell wall polymers. Five spectra were averaged from two CaF₂ windows and two different stem sections were used. The second derivative of the spectra was computed using the well-known Savitsky-Golay algorithm to extract more spectral features (41).

RESULTS

Protein Identification and Functional Classification—Three different extraction protocols were used to obtain fractions enriched in soluble proteins (S), cell wall proteins (C) and membrane proteins (M) from 4 different flax organs/tissues (outer- and inner-stems, leaves and roots, supplemental Fig. S1). Trypsin-digested peptides from the 24 different samples (12 samples × 2 biological replicates) were then individually analyzed by liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) and proteins identified using the sequenced flax genome as reference (3). Only those proteins (2355) present in both biological replicates were retained for further analyses. Of these 2355 proteins, 1242 were nonredundant (supplemental Data S1 and S2). Two hundred and nineteen proteins were uniquely found in the S fraction, 33 in the C fraction and 740 in the M fraction (Fig. 1A). Two hundred and twelve proteins were common to 2 fractions and 38 proteins were common to all 3 fractions (Fig. 1A). The use of specific extraction protocols in addition to the soluble fraction step enabled us to recover 62 (cell wall fraction) and 769 (membrane fraction) additional proteins, of which 29 proteins were common to the C and M fractions. One hundred and sixteen, 100, 189, and 186 proteins (all fractions) were uniquely identified in leaves, roots, outer-stems and inner-stems respectively (Fig. 1B). In all organs/tissues, 322, 196, and 133 proteins were common to 2, 3, or all 4 samples (Fig.

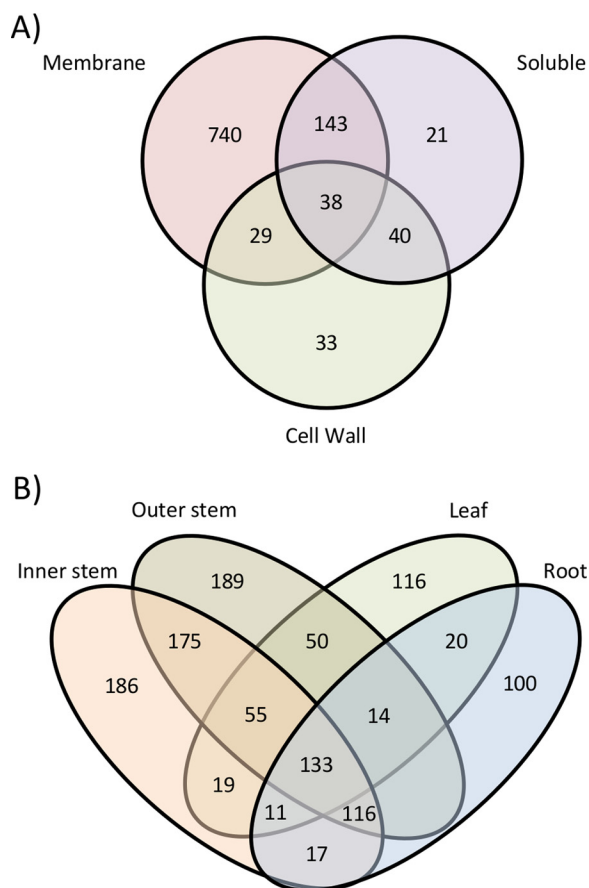


FIG. 1. Repartition of total nonredundant proteins identified in 3 cellular compartments (A) and 4 plant organs/tissues (B). Fractions enriched in soluble proteins (S), membrane proteins (M) and cell wall proteins (C). Inner stem (IS), outer stem (OS), leaves (L) and roots (R).

1B). 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), glycosyl transferases (GT), 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 1, 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, CBL, and EXPN CAZy 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

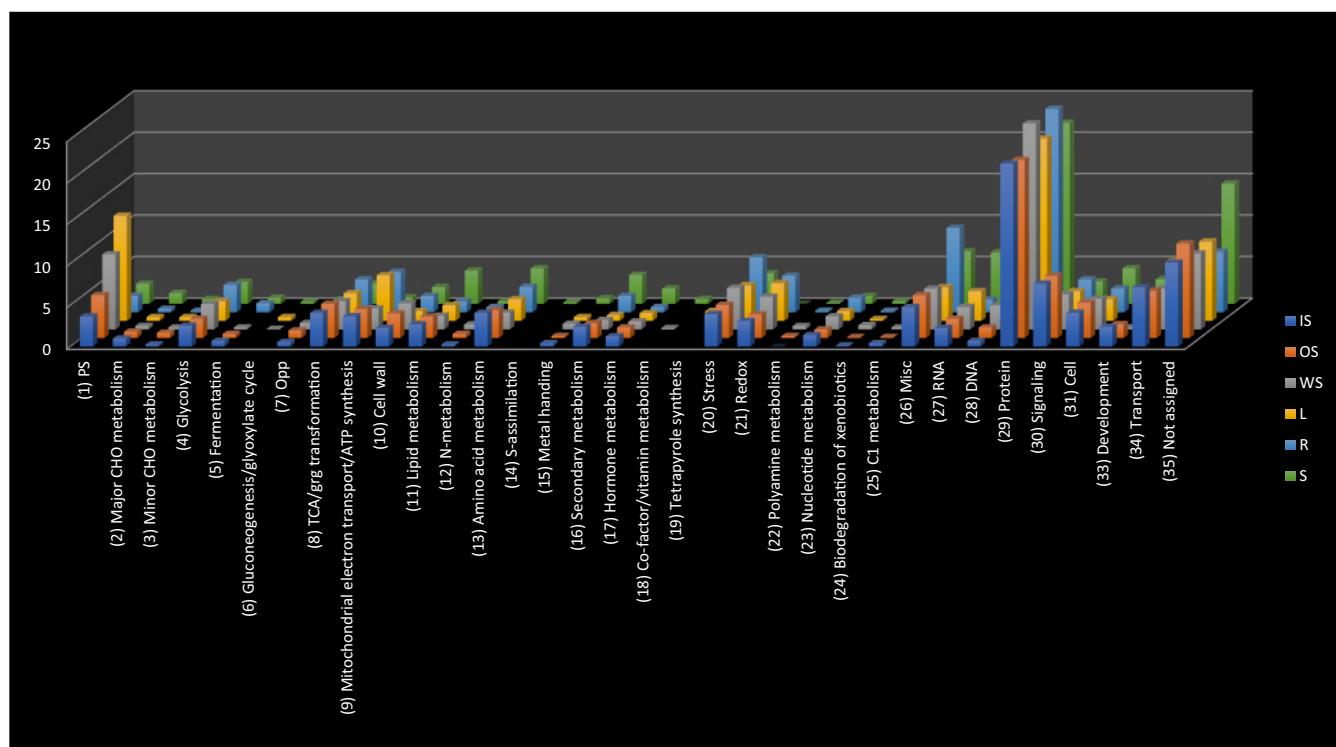


FIG. 2. **Functional classification of flax proteins.** Histogram showing repartition of flax proteins from 6 different organs/tissues in Mapman functional classes. IS: Inner stem; OS: Outer stem; WS: Whole stem; L: Leaves; R: Roots; S: Seeds.

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 flax cell wall proteome. Many of these proteins are physically located in the cell wall and/or apoplast and are therefore secreted. The proteins were identified using several criteria (presence of peptide signal, secretion predicted by TargetP, SignalP and Predotar software, no more than 1 transmembrane domain). Altogether 355 proteins (including 103 CAZy proteins) representing 11.18% total pro-

teins satisfied these criteria and were added 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

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) McFarlane 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
<i>Glycoside hydrolases</i>				
GH1* (15)	β -glucosidase,	Glucan, cellulose, hemicellulose (xyloglucan)	1,2	All
GH3* (12)	β -xylosidase, α -arabinofuranosidase	Hemicellulose (xylan)	1,2	All
GH5_7 (2)	β -mannase	Mannan	1,2	WS, S
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)	glucanase	MLG, Callose	1,2	WS, L, R, S
GH28 (4)	Polygalacturonase, pectinase	Pectin (homogalacturonan)	1,2	IS, WS
GH31* (4)	α -glucosidase, α -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), AGPs	2,4	S
<i>Glycosyltransferases</i>				
GT1 (12)	UDP-glycosyltransferase	Lignin (monolignols)	5	IS, S
GT2* (3)	Cellulose synthase, cellulose-synthase-like (mannan synthase, glucomannan synthase, glucan synthase), galactosyltransferase	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, galactosyl transferase, galacturonosyltransferase	Hemicellulose: Pectin (RGI), xyloglucan, xylan	7,8,9	IS, OS
GT48 (1)	Glucan synthase	Callose	8	S
GT75* (4)	Glucuronosyltransferase	Hemicellulose (xylan)	10	WS, S
GT77 (1)	Xylosyltransferase, Arabinosyltransferase,	Pectin (RGII), AGP	4,9	IS
<i>Auxilliary activity</i>				
AA1 (11)	Phenoloxidase/Laccase	Lignin	11	IS, OS, S
AA2 (40)	Phenoloxidase/Peroxidase	Lignin		All
<i>Carbohydrate esterases and Polysaccharide lyases</i>				
CE8 (8)	Pectin methylesterase	Pectin	2	OS, WS, L, R, S
CE13 (2)	Pectin acetyesterase	Pectin	2	OS, R
PL4_2 (1)	Pectate lyase	Pectin		OS
<i>Carbohydrate Binding Modules and Expansin</i>				
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

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 II

Amino acid sequences in (bold text) and around the loop involved in the specific hydrolytic activity for the class IIIA XTH proteins of *L. usitatissimum*, and *A. thaliana* (selected sequences)

Gene ID	Sequence	Sequence
<i>A. thaliana</i> _19659993	QTNVFVRGSGD	- R N V I G
<i>L. usitatissimum</i> _23180042	QTNVYVRGSGD	- G N I I G
<i>L. usitatissimum</i> _23152616	QTNVYVRGSGD	- G N I I G
<i>L. usitatissimum</i> _23171810	QTNVYVRGSGD	- R N V V G
<i>L. usitatissimum</i> _23179194	QTNVYVRGSGD	- R N I V G
<i>L. usitatissimum</i> _23159458	QTNVYVRGSGD	G G D I I G
<i>L. usitatissimum</i> _23164289	QTNVYVRGSGD	G G D I I G
<i>A. thaliana</i> _19639833	QTNVYIRGSGD	- G K I I G
<i>L. usitatissimum</i> _23159481	QTNVYIRGSGD	- G K I I G
<i>L. usitatissimum</i> _23164234	QTNVYIRGSGD	- G K I I G
<i>L. usitatissimum</i> _23146127	QTNVYIRGSGD	- G R I I G
<i>L. usitatissimum</i> _23169892	QTNVYIRGSGD	- G R I I G
<i>L. usitatissimum</i> _23154089	QTNLYGNGSTH	- - - - R G
<i>A. thaliana</i> _19654973	QTNLYGNGSTH	- - - - L G
<i>L. usitatissimum</i> _23151771	QTNLYGNGSTN	- - - - L G

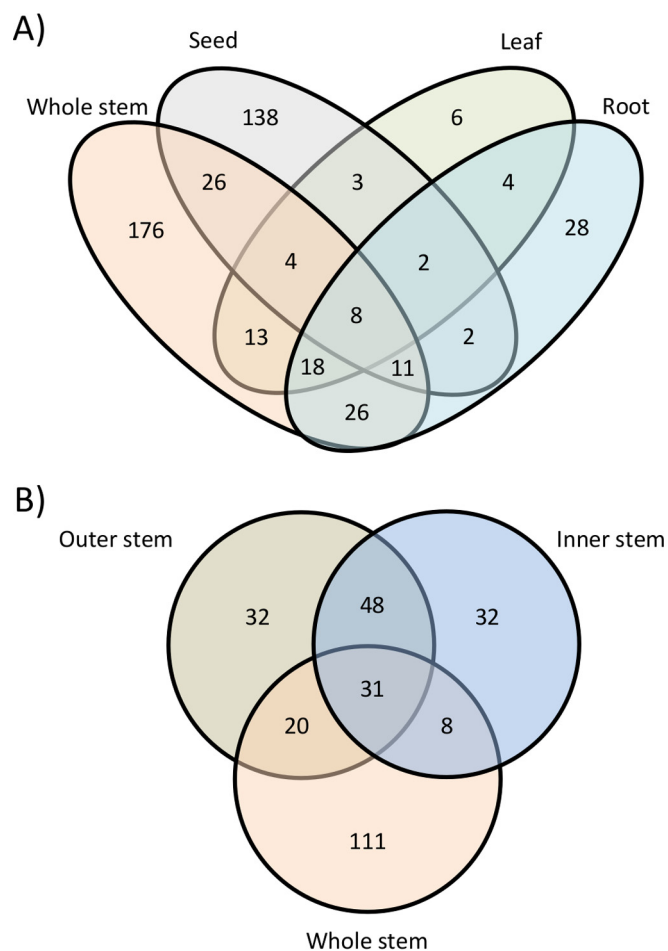


FIG. 3. Repartition of the 465 flax Total CWPs identified in 4 different organs (A) and stem tissues (B). Whole stem (WS), Seeds (S), leaves (L), roots (R), Inner stem (IS), Outer stem (OS).

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. 7A, 7C). Although no labeling was observed in bast fiber cell walls with LM10 antibodies (Fig. 7B), a relatively intense labeling was present in the middle lamella/primary cell wall of bast fibers with LM11 antibodies that recognize more heavily substituted xyans. The middle lamella/primary cell wall of bast fibers was heavily labeled by the xyloglucan antibodies LM15 and LM24 that recognize the XXXG and XLXG/XXLG oligosaccharide epitopes, respectively (Fig. 7D, 7E). LM15 antibodies also recognized epidermal cell walls and gave a weak labeling in xylem walls (Fig. 7D). The LM24 antibodies weakly labeled xylem and pith cell walls (Fig. 7E). Finally, the LM21 antibody that recognizes mannans/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. 7F). Overall these results indicate that flax fiber cell walls

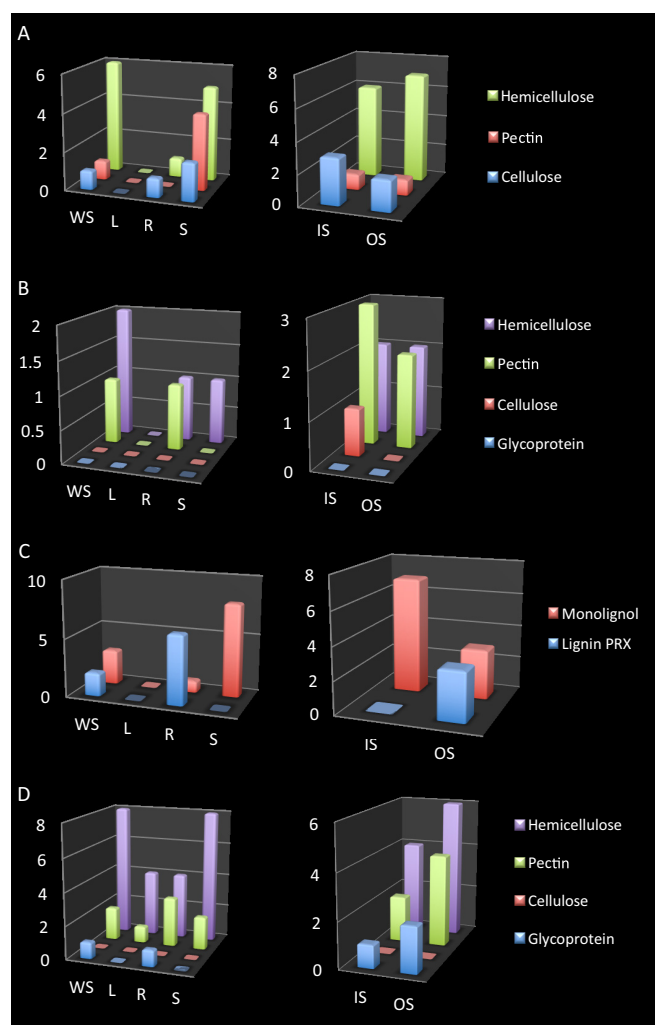


FIG. 4. Number of proteins and protein families involved in cell wall polymer biosynthesis (A–C) and polymer remodeling (D) in whole stems (WS), leaves (L), roots (R), seeds (S), inner stems (IS) and outer stems (OS). A, Number of different enzymes involved in UDP-sugar production associated with different cell wall polymers; B, Number of different CAZy families involved in polysaccharide polymer biosynthesis; C, Number of canonical lignin (monolignol) enzyme families and lignin-related peroxidases, D, Number of different CAZy families involved in polysaccharide polymer remodeling.

contain different types of hemicelluloses: xyloglucans (middle lamella, primary cell wall), substituted xylans (middle lamella/primary cell wall) and mannans/heteromannans (secondary cell wall/G-layer). The presence of these hemicellulose types in bast fiber cell walls was also confirmed by FT-IR microspectroscopy that enabled us to identify significant bands characteristic of xylans ($1313, 1452\text{ cm}^{-1}$), arabinoxylans (1053 cm^{-1}), xyloglucans ($1201, 1313, 1371, 1452\text{ cm}^{-1}$) and mannans (1053 cm^{-1}) (Fig. 8). The presence of other characteristic bands also indicated the presence of cellulose ($1429, 1201, 1161, 1103\text{ cm}^{-1}$), pectin ($1275, 983\text{ cm}^{-1}$) and (lignin (1514 cm^{-1}) (supplemental Fig. S10).

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: XLFG). These fragments are derived by enzymatic cleavage of nonsubstituted glucose residues of a fucosylated XXXG-type xyloglucan. Ions corresponding to the previously identified glucuronoxylan oligosaccharide (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-

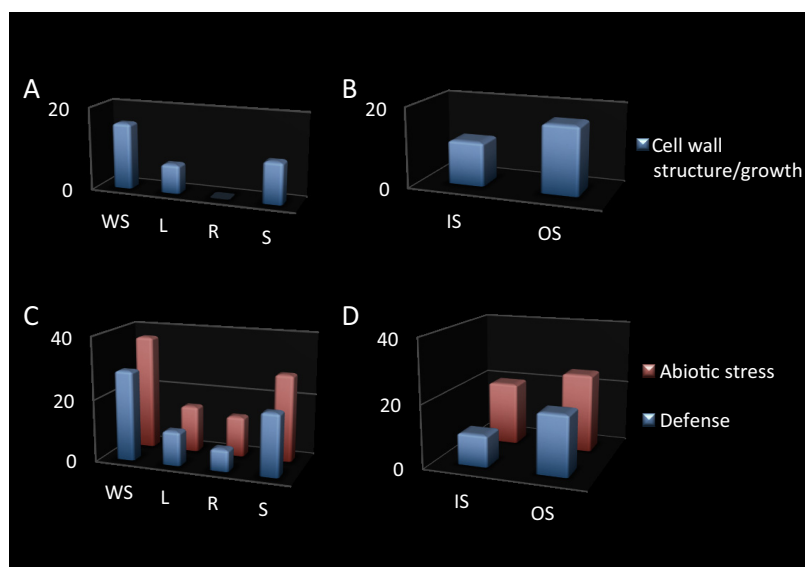


FIG. 5. Number of proteins involved in cell wall structure/growth (A, B) and defense/stress (C, D) in whole stems (WS), leaves (L), roots (R), seeds (S), inner stems (IS) and outer stems (OS).

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

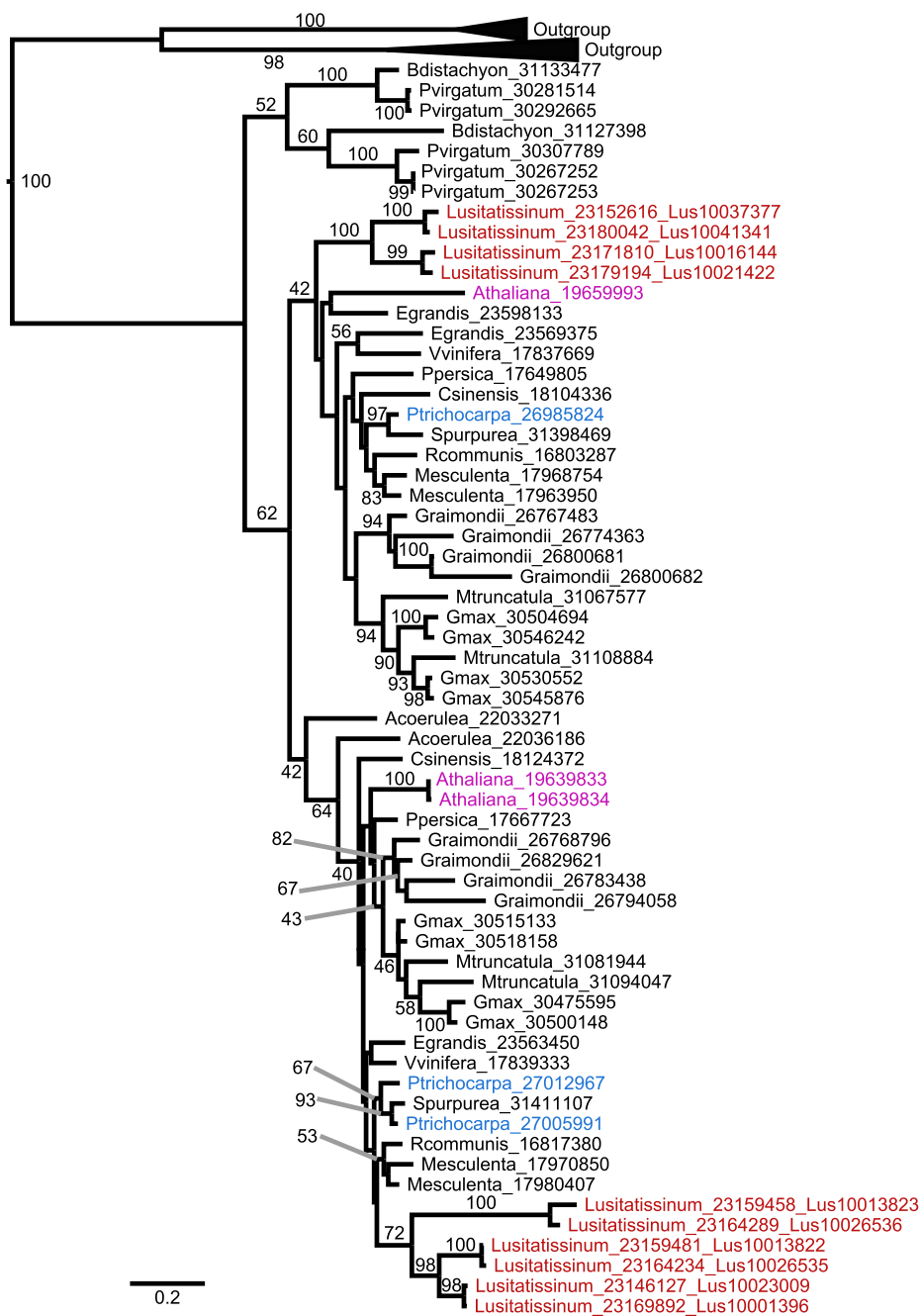


FIG. 6. Maximum likelihood phylogenetic tree of the xyloglucan endo-transglycosylase/hydrolase family IIIA created using a selection of plant species present in Phytozome database v10. Branch support values obtained with 1000 bootstrap repetitions. The tree is rooted with the non-III A sequence. The scale bar shows the inferred number of amino acid substitutions per site. Protein sequences found in *Linum usitatissimum*, *Populus trichocarpa*, and *Arabidopsis thaliana* are colored in red, blue and purple, respectively.

production. 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 mechan-

ical 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

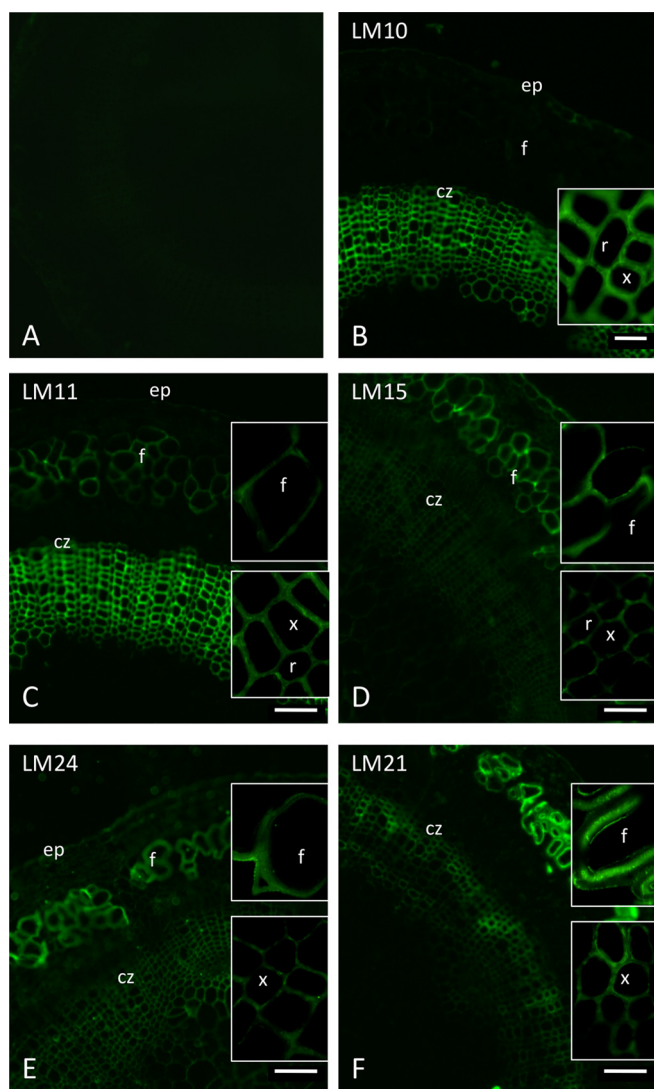


FIG. 7. Immunolocalization of flax stem hemicellulose epitopes. Pre-immune serum (A); anti-xylan LM10 antibodies (B); anti-xylan arabinoxylan LM11 antibodies (C); anti-xyloglucan LM15 antibodies (D); anti-xyloglucan LM24 antibodies (E); anti-mannan/heteromannan LM21 antibodies (F). cz: cambial zone;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

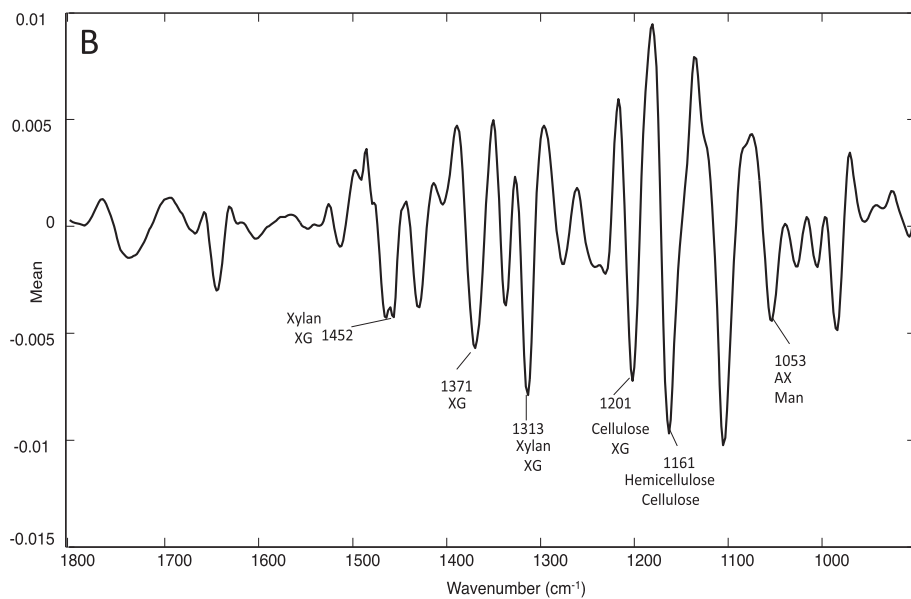
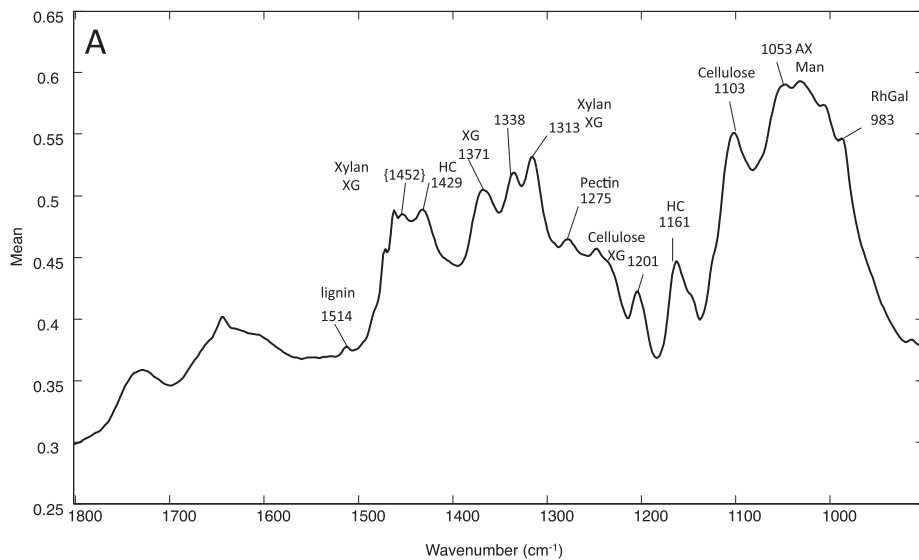
nonlignin phenylpropanoids responsible for UV protection, as well as limited lignification in these tissues (20, 62). Monoglucan biosynthetic enzymes were also present in the seed cell wall proteome where they are probably related to the biosynthesis of lignans, biologically active dimers of monoglucans (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).

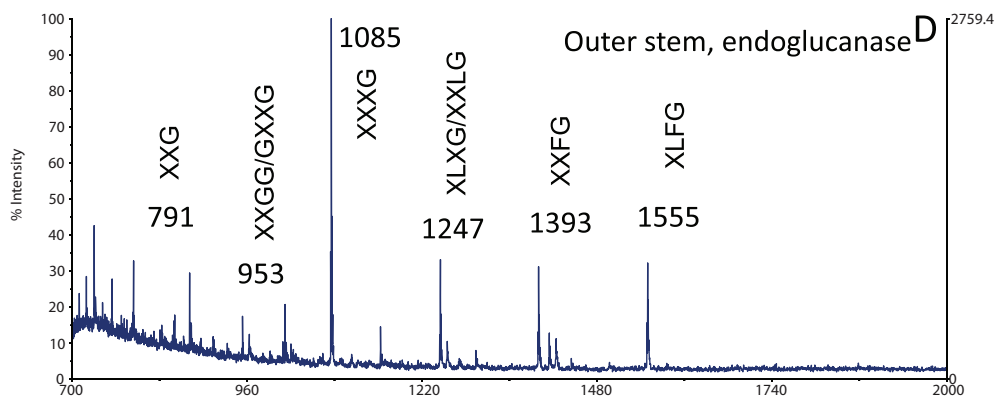
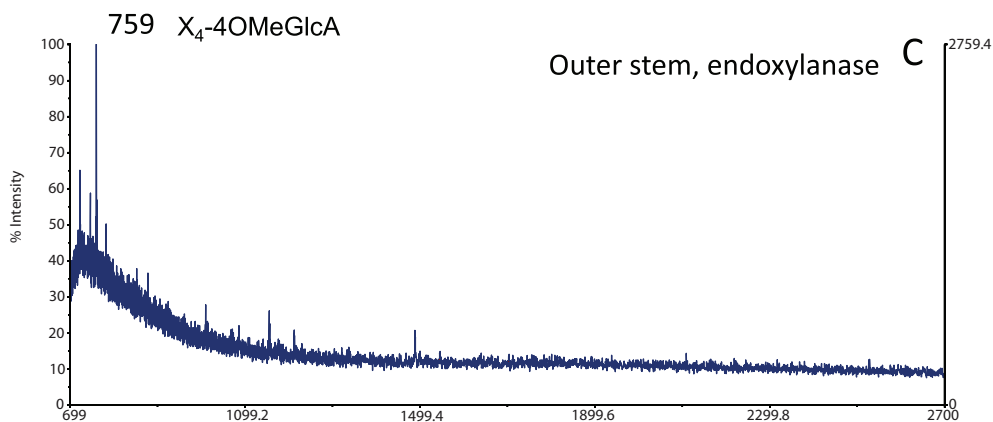
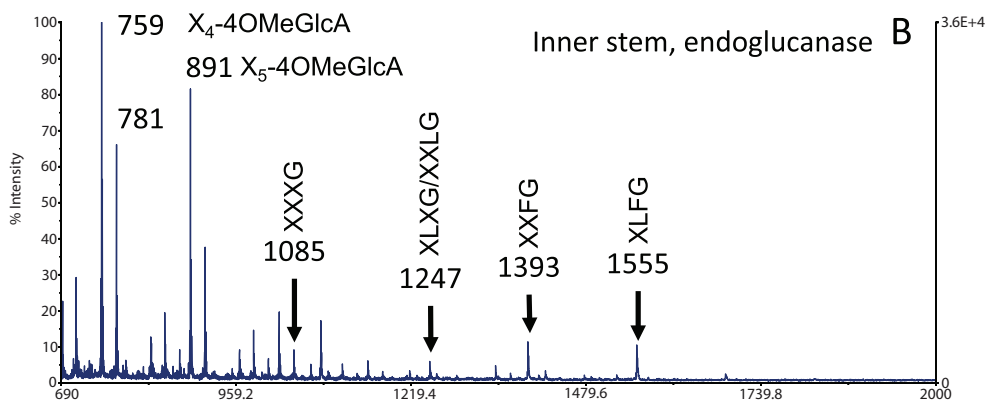
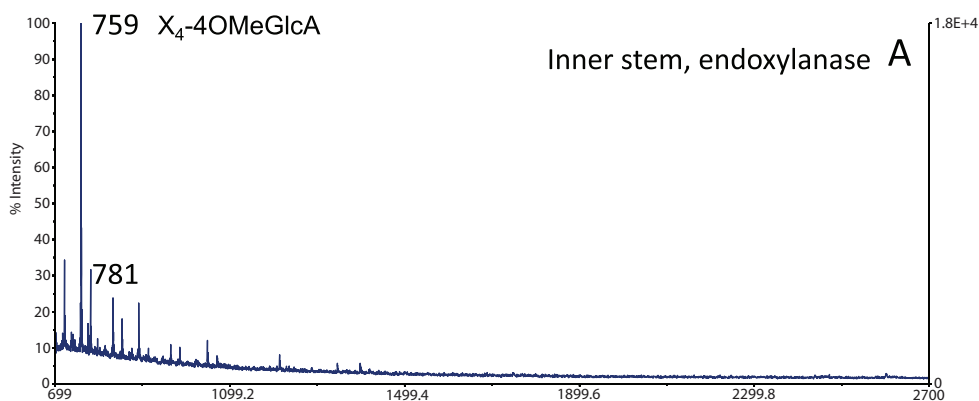
Cell walls are also an important interface between the plant and its environment, and cell wall proteins therefore play a nonnegligible role in responses toward abiotic stress and pathogens (biotic stress). Analysis of cell wall proteins involved in abiotic stress responses indicated that proteins belonging 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).



C

Wavenumber (cm-1)	Cell Wall polymers
{1452}	Xylan, Xyloglucan (XG)
1371	Xyloglucan
1313	Xylan, Xyloglucan
1201	Cellulose, Xyloglucan
1161	Hemicellulose, Cellulose
1053	Arabinoxylan (AX), Mannan (Man)

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 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 maturing 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-xyloglucans 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 endoxylanase 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 xyloglucans 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 Tm-NXG1 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: X₄-4OMeGlcA + Na⁺, 781: X₄-4OMeGlcA + 2Na⁺ - H⁺, 891: X₅-4OMeGlcA + 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 xylans, 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 macrofibrils (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).

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