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# Profiling the main cell wall polysaccharides of tobacco leaves using high-throughput and fractionation techniques

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ABSTRACT

Nicotiana species are used to study agriculturally and industrially relevant processes, but limited screening methods are available for this species. A tobacco leaf cell wall preparation was fractionated using both chemical and enzymatic methods; the fractions obtained were subsequently analysed using rapid high-throughput wall profiling tools. The results confirmed previous data showing that mature tobacco leaf cell walls are predominantly composed of pectic homogalacturonans with lesser amounts of hemicellulosic arabinoxyloglucan and glucuronoxylan polymers. This confirmation provided proof that the profiling methods could generate good-quality results and paves the way for high-throughput screening of tobacco mutants where a range of biological processes, where the cell wall profile is important, are studied. A novel enzymatic oligosaccharide fingerprinting method was optimized to rapidly analyse the structure of XXGG-rich arabinoxyloglucans characteristic of Solanaceae species such as tobacco. Digestion profiles using two available xyloglucanase-specific endoglucanases: *Trichoderma reseei* EGII and *Paenibacillus* sp. xyloglucanase were compared showing that the latter enzyme has a higher specificity toward tobacco arabinoxyloglucans, and is better-suited for screening. This methodology would be suitable for species, such as tomato (*Solanum lycopersicum*) or potato (*Solanum tuberosum*), with similar XXGG-type motifs in their xyloglucan structure.



## 1. Introduction

The composition and structure of plant cell wall polysaccharides are of interest since they constitute some of Nature's most abundant polymers and are important resources (i.e. raw materials such as wood and coal) (Albersheim, Darvill, Roberts, Sederoff, & Staehelin, 2011). Plant cell walls are also integral to the interaction of the plant body and its various organs and tissues with the complex external environment (biotic and abiotic). Plant cell walls have been extensively characterised and are known to be dynamic matrix structures with some species-specific characteristics (Fry, 2011). Although the general cell wall characteristics for several plant species are known, few important crop species and research model plants have yet benefited from the numerous tools available to rapidly profile their cell wall polysaccharides. The primary cell wall polysaccharides of tobacco vegetative tissues are of interest because *Nicotiana* spp. are used as scientific model systems to study a number of important agriculturally and industrially relevant processes. Examples of these include; (i) the mechanisms involved in disease resistance to pathogen infection (e.g. to fungi such as *Botrytis cinerea*) (Capodicasa et al., 2004), (ii) the *in planta* effects of over-expressing or silencing genes in plant biotechnology strategies, molecular farming (Lupotto & Stile, 2007), and (iii) the ability to bioengineer plants with improved cellulose extractability for fibre production, efficient pulp and paper processing (Bindschedler et al., 2007) and to provide ready utilizable biomass for biofuel applications (Wang et al., 2011).

The cell wall polysaccharides of *Nicotiana tabacum* and *Nicotiana plumbaginifolia* cell suspensions and vegetative tissues have, over the last three decades, already been analyzed with classical carbohydrate chemistry approaches (classical polymer isolation and characterization studies) (Eda and Kato, 1980; Iraki, Bressan,

*Abbreviations:* AIR, alcohol insoluble residue; CoMPP, comprehensive microarray polymer profiling; CBM, carbohydrate binding module; mAb, monoclonal antibody; EPG, endopolygalacturonase; ESI-MS, electrospray ionization–mass spectrometry; FT-IR, Fourier transform–infrared spectroscopy; HPAEC-PAD, high performance anion exchange chromatography-pulse amperometric detection; PMAA, partially methylated alditol acetate; XEG, xyloglucan-specific endoglucanase; RG, rhamnogalacturonan; HG, homogalacturonan; XyG, xyloglucan; AXyG, arabinoxyloglucan; *Psp*XEG5, *Paenibacillus* sp xyloglucan specific endoglucanase 5; *Tr*EGII, *Trichoderma reseei* endoglucanase II.

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& Carpita, 1989; Sims, Munro, Currie, Craik, & Bacic, 1996). The goal of many of these early studies was to isolate purified polysaccharides for in-depth structural characterization rather than to provide a holistic overview of the cell wall structural composition. For example it was shown that HG-rich pectin is the main polysaccharide present in tobacco mesophyll cells and midribs (Eda and Kato, 1980; Eda, Miyabe, Akiyama, Ohnishi, & Kato, 1986). An alkaline pectin fraction was also found in midribs and shown to contain RG polymers (Eda, Kato, Ishizu, & Nakano, 1982). Apart from being rich in pectin polymers, an unusual feature of tobacco cell wall structure is the nature of their xyloglucan (XyG) polysaccharides (Eda & Kato, 1978). Sims et al. (1996) and York, Kumar Kolli, Orlando, Albersheim, & Darvill (1996) provided structural analyses from tobacco cell suspension and extracellular polysaccharides (*N. tabacum* and *N. plumbaginifolia*) showing that the arabinoxyloglucan (AXyG) structure of tobacco displays an unusual XXGG repeat motif in its structure, compared to the XXXG unit present in *Arabidopsis thaliana* (Brassicaceae family) cell walls (see Vincken, York, Beldman, & Voragen, 1997). This unusual XXGG pattern was shown to be common to the Solanaceae family, which also include tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*).

In the 1970s and 1980s a variety of biochemical and physiological methods and techniques were developed to analyze and characterize plant cell walls (Fry, 1988). Later, some of these traditional methods were adapted to screen/profile polysaccharide-rich walls of mutant or transgenic plant lines. Zablackis, Huang, Müller, Darvill, & Albersheim (1995) provided the first model plant reference dataset for *Arabidopsis*, performing a complete characterization of leaf cell wall polysaccharides of this model species. Most, if not all of these wall profiling techniques have been originally optimized to study this model plant species. For instance, Reiter and coworkers (Reiter, Chapple, & Somerville, 1993; Reiter, Chapple, & Somerville, 1997) used a known gas liquid chromatography method for the determination of cell wall monosaccharide composition (Blakeney, Harris, Henry, & Stone, 1983) to screen *Arabidopsis* cell wall mutants. Other approaches based on cell wall immunochemistry (Hervé, Marcus, & Knox, 2011; Knox, 1997) and Fourier transformed infrared spectroscopy (FT-IR; Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000) were similarly used to rapidly screen *Arabidopsis* cell wall mutants (for FT-IR-based screening, see Mouille, Robin, Leconte, Pagant, & Höfte, 2003; for immunochemistry-based approach, see Moller et al., 2007). Moreover, during the last decade, the availability of cell wall degrading enzymes, combined with different analytical techniques provided improved methods to screen and analyze plant cell wall mutants. For instance, XyGs of *Arabidopsis* cell wall-deficient mutants were profiled using a xyloglucan-specific endoglucanase (XEG; Lerouxel et al., 2002; Nguema-Ona et al., 2006, Persson, Sorensen, Moller, Willats, & Pauly, 2011).

In order to provide tools to rapidly analyze and profile the cell wall composition and structure of fully expanded mature tobacco leaves, and in the context of the importance of *N. tabacum* as a model plant system to study a number of processes where cell walls play a key role, we have performed a thorough profiling analysis of the pectin and hemicellulosic polymers present in tobacco leaves. To generate these reference data profiles, we have used a combination of high-throughput techniques including monosaccharide compositional analysis, FT-IR spectroscopy, comprehensive microarray polymer profiling (CoMPP) analysis and oligosaccharide mass fingerprinting as well as more in-depth approaches using chemical and enzymatic fractionation methods to validate the screening tools. We propose that this combination of tools provide a ready-to-use approach for plant biologists in general who need to profile the different wall polymers present in the leaves of *N. tabacum* plant populations. In particular, the use of *Psp*XEG5 was shown to be highly specific for arabinoxyloglucan and in combination with monosaccharide composition and/or linkage analysis could provide an efficient screening method for Solanaceae species such as tobacco, potato and tomato.

## 2. Experimental

### 2.1. Plant material

Tobacco seeds (background *N. tabacum* L. Havana petit SR1) were sown on solid MS media (Murashige & Skoog, 1962) in petri dishes and incubated at 26 °C with a 16 h light/8 h dark photoperiod regime. Six weeks old seedlings were transferred to soil and grown in a climate room (23 °C; 16 h light 8 h dark photoperiod; for 8–12 weeks) until fully mature plants (i.e. 8 leaf stage) were available. Fully expanded leaves were harvested and flash-frozen using liquid nitrogen and stored at −80 °C until further use. For statistical purposes four biological samples (i.e. leaves) per analyses were utilised with two technical repeats per sample.

### 2.2. Cell wall isolation and fractionation

Cell wall materials were extracted from frozen tobacco leaves and fractionated according to a protocol modified from Nguema-Ona et al. (2006). Briefly, 2 g of frozen leaves were ground, under liquid nitrogen using a mortar and pestle, to a fine powder. After boiling in 80% ethanol for 20 min, insoluble material was washed in methanol: chloroform (1:1) for 24 h, and this was performed with fresh solvents for an additional 24 h due to the high lipid/oil content, thereafter the residue was washed in methanol before air drying. The dry material, referred to as alcohol insoluble residue (AIR), was de-starched using a combination of thermostable α-amylase, amyloglucosidase and pullulanase (all from Megazyme; see Harholt et al., 2006). De-starched AIR was both chemically (adapted from Schols & Voragen, 2003) and enzymatically fractionated. Chemically extracted fractions were dialyzed (3.5 kDa cutoff dialysis tubing) against deionized water (48 h at 8 °C), freeze dried before gravimetric, FT-IR spectroscopic and compositional analyses were conducted. The 4 M KOH soluble fraction was also digested with XEGs to obtain an XEG-soluble fraction for compositional analysis. Enzymatic fractionation (see Fig. 2B) was performed with glycosyl hydrolases (GHs) sourced from Megazyme, an *Aspergillus niger* endopolygalacturonase (EPG) (E-PGALS; GH family 28) for pectin digestion, and two different xyloglucan-specific endoglucanases (XEG): an endoglucanase-II from *Trichoderma longibrachatum*, formerly known as *T. reseei* (E-CLTR; GH family 74; Grishutin et al., 2004; Foreman et al., 2003) and a recombinant XEG from *Paenibacillus* sp (E-XEGP; GH family 5; Yaoi, Nakai, Kamed, Hiyoshi, & Mitsuishi, 2005). All digestions were performed at 37 °C for 16 h. EPG-soluble fractions were dialyzed (3.5 kDa cutoff dialysis tubing) against deionized water (48 h at 8 °C), freeze dried prior to analysis. XEG-soluble fractions were either filtered (0.22 μm nylon membrane) prior to analysis by HPAE chromatography and/or methylation analyses, or further desalted and concentrated as described by Packer, Lawson, Jardine, & Redmond (1998), by using a graphitised solid phase extraction (UltraClean SPE columns; Altech, USA) column and a Visiprep vacuum manifold (Supelco, USA). SPE columns were conditioned with 4 mL of 90% aqueous (v/v) acetonitrile in 0.1% aqueous (v/v) trifluoroacetic acid (TFA) and with 4 mL of distilled water. 1 mL of extract was applied to the equilibrated column and then washed with 4 mL of deionised water after adsorption. To elute oligosaccharides, the columns were rinsed with 4 mL of 25% aqueous

(v/v) acetonitrile in 0.1% (v/v) TFA. The fractions were finally freeze-dried and re-suspended in deionised water before further analyses were performed using mass spectrometry and gas liquid chromatography.

### 2.3. Monosaccharide composition analysis by gas chromatography

A gas liquid chromatography method (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) was used to determine the monosaccharide content of cell wall residues and fractions. Approximately 1–3 mg of wall residue or fractionated material was hydrolyzed (2 M TFA, 110 °C, 2 h) and the liberated monosaccharides converted to methoxy sugars using 1 M methanolic HCl at 80 °C for 24 h. Silylation was performed at 80 °C (20 min) to produce trimethyl-silyl-glycosides which were dissolved in cyclohexane. The derivatives were separated and analyzed in a gas chromatograph (Hewlett Packard 5890 series II) coupled to a flame ionization detector, using a 30 m × 0.25 mm (i.d.) HPS-MS column. The oven temperature program was stabilised at 120 °C for 2 min, ramped at 10 °C/min to 160 °C, then at 1.5 °C/min to 220 °C and finally at 20 °C/min to 280 °C. Myo-inositol (0.5 μmol) was used as the internal standard. Derivatives were identified based on their retention time and quantified by determination of their peak areas. Monosaccharides (from Sigma–Aldrich) were used as standards to determine the retention time of the nine main monosaccharides found in plant cell walls. The sugar composition was expressed as mole percentage of each monosaccharide. Error bars in the histograms represent the standard deviation (SD) of the mean of four biological samples with two technical replicates per biological sample.

### 2.4. Methylation analysis of XEG-soluble fractions

Tobacco 4 M KOH soluble fraction and XEG-soluble fractions were methylated by the NaOH method of Ciucanu and Kerek (1984). Freeze-dried fractions (1–2 mg) were solubilized in 500 μL of DMSO, stirred overnight and sonicated prior to the start of methylation. Two to three NaOH pellets were ground to powder and two small spatula scoops were added to a borosilicate glass test tube, and the contents vigorously mixed. Two 25 μL portions of methyl iodide were added 20 min apart, followed after 20 min by a third addition of 50 μL and the mixture was stirred for 2 h. The reaction mixture was quenched with 1 mL 100 mg/mL freshly prepared sodium thiosulfate. One mL of dichloromethane was immediately added to the test tube and vigorously mixed to form an emulsion. After a brief centrifugation (30 s at 5000 rpm), the upper aqueous phase was removed and the lower phase washed three times with water before drying under a stream of nitrogen. This first methylation step was repeated once. The methylated polysaccharides obtained were hydrolysed at 110 °C for 90 min in 0.5 mL of 2 M trifluoroacetic acid and the hydrolyzed samples were dried in a SpeedVac at 50 °C, re-suspended in 100 μL of propan-2-ol before re-drying. The partially methylated monosaccharides were dissolved in 100 μL of 1 M $NH_4OH$ and 500 μL of 20 mg/mL $NaBD_4$ in DMSO were added to the mixture. This reduction reaction was performed overnight at room temperature. Excess reductant was destroyed with 100 μL of glacial acetic acid. 100 μL of 1-methyl imidazole and 0.5 mL of acetic anhydride were successively added to the mixture. Acetylation of the partially methylated alditols was performed at room temperature for 15 min. Unreacted anhydride was destroyed with 1.5 mL of $H_2O$, and the partially methylated alditol acetate (PMAA) derivatives were partitioned into 1.0 mL of dichloromethane, washed five times with water, and dried. Finally, the partially methylated alditol acetates derivatives were dissolved in 200 μL dichloromethane, and analyzed using a gas chromatograph (Agilent 6890N) linked to a mass spectrometer (Agilent 5975 inert mass selective detector), using a 30 m × 0.25 mm (i.d.) RTX 5 SIL-MS column. The oven temperature program was stabilised at 50 °C for 2 min, and successively ramped at 40 °C/min to 130 °C and 4 °C/min to 250 °C. Identification of the PMAA derivatives and deduction of their glycosidic linkages was achieved using a combination of reference data available publicly at (http://www.ccrc.uga.edu/specdb/ms/pmaa/pframe.html), from published PMAA spectra (Carpita & Shea, 1989), and from total ion chromatogram (TIC) data and derived mass spectra of both a purified mixture of tamarind xyloglucan oligosaccharides and β-1,4-xylotetraose (both from Megazyme).

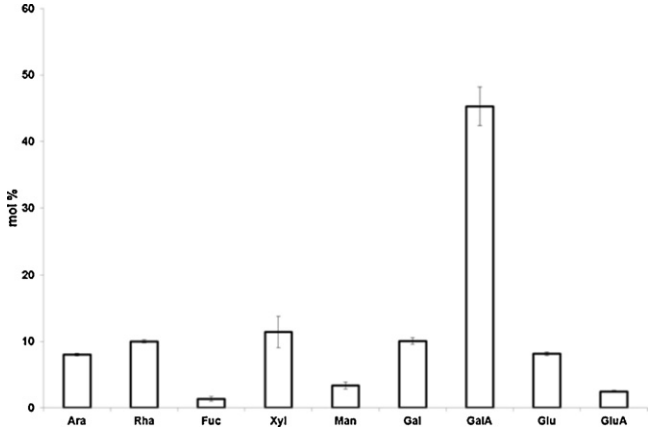


**Fig. 1.** Monosaccharide compositional analysis (expressed in mol%) of total (de-starched) alcohol insoluble residue prepared from fully expanded tobacco leaves. Ara, arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid.

### 2.5. High performance anion exchange chromatography

XEG-generated arabinoxyloglucan oligosaccharides were analysed by high performance anion exchange chromatography (HPAEC) using Dionex Ultimate 3000 series chromatograph with pulsed amperomertric detection (PAD, Coulochem III electrochemical detector) and a CarboPac PA1 column (4 mm × 250 mm) and precolumn (4 mm × 50 mm). Samples (10 μL) were eluted at 1 mL/min using the following sodium acetate gradient in 100 mM NaOH: 0–2.5 min: 100 mM NaOH, 2.5–3 min: linear gradient of 0–70 mM NaOAc, 3–29 min: linear gradient of 70–100 mM NaOAc, 29–29.1 min: linear gradient 100–500 mM NaOAc, 29.1–38 min, 500 mM NaOAc, 38–47 min, 100 mM NaOH.

### 2.6. Electrospray ionisation–mass spectrometry

The electrospray ionization mass spectrometry (ESI-MS) was performed on a Waters QTOF OPTIMA (Milford, MA, USA). Samples (1 μL) were directly injected into a stream of 80% acetonitrile, 0.1% formic acid using a Waters UPLC at flow rate of 0.2 mL/min. The ion source was set as follows: Source Electrospray positive; Capillary voltage 3 kV; Cone Voltage 40 V. Leucin enkaphalin was used as the lock mass. The mass spectrometer was calibrated with sodium formate.

### 2.7. Infra-Red (IR) spectroscopy of cell wall fractions

A NEXUS 670 FTIR instrument containing a Golden Gate Diamond ATR accessory with a type IIa diamond crystal was used for ATR-FT-IR measurements. The spectra were recorded between 4000 and 650 $cm^{-1}$ with a Geon-KBr

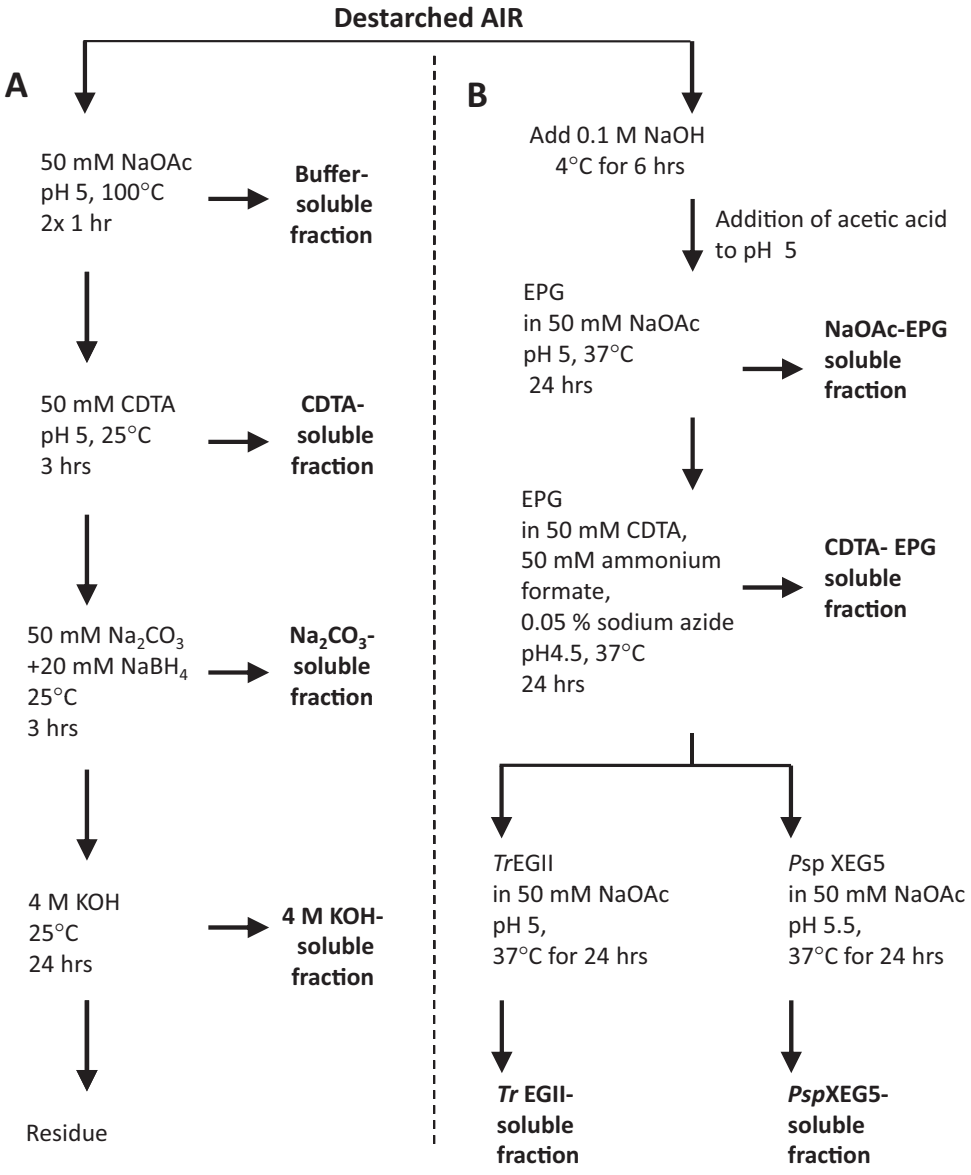


**Fig. 2.** Flow diagram of the chemical fractionation procedure (A) and enzymatic fractionation procedure (B) performed on alcohol insoluble residue prepared from tobacco leaves. EPG: endopolygalacturonase, *Tr*EGII: *Trichoderma reseei* endoglucanase II, *Psp*XEG5: *Paenibacillus* sp. xyloglucan-specific endoglucanase 5.

beamsplitter and DTGS/CsI detector. Spectral data (128 co-added scans per sample) were processed using Unscrambler™ (Camo® Inc. USA). The spectral region (600–4000 $cm^{-1}$) displayed was limited to the wall protein, lipid and carbohydrate regions (700–2000 $cm^{-1}$).

### *2.8. Comprehensive microarray polymer profiling (CoMPP) analysis of cell wall material*

AIR was prepared from frozen tobacco leaves as described in the 'cell wall isolation and fractionation' section of this paper. The chemical fractionation process was simplified to three extraction steps. First an aqueous CDTA extraction was performed on the AIR followed by a NaOH extraction. Finally a cadoxen extraction was applied which facilitated the partial solubilisation of the remaining cellulose-rich residue. Approximatively 40 mg of material was used to perform the CoMPP analyses as described in Moller et al. (2007). The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data set was set to 100 and all other data adjusted accordingly. A cut off value of 5 was imposed.

## 3. Results and discussion

Monosaccharide composition of the total tobacco leaf cell wall material was determined by gas chromatography analysis of the soluble supernatant obtained from TFA hydrolysis of AIR. Examination of the total monosaccharide composition of tobacco leaf cell walls (Fig. 1) reveals an abundance of GalA (ca. 45%) which would indicate a predominance of pectin polymers. Eda and Kato (1980) and Eda et al. (1986) analysed *N. tabacum* mesophyll cells and the cell wall composition of leaf midribs and also found a predominance of pectin polymers. The presence of Rha, Ara and Gal at lower levels suggests that the main pectin polymers; rhamnogalacturonan-I (RG-I), rhamnogalacturonan II (RG-II), and homogalacturonan (HG), are present. However, the rather high level of GalA in the total

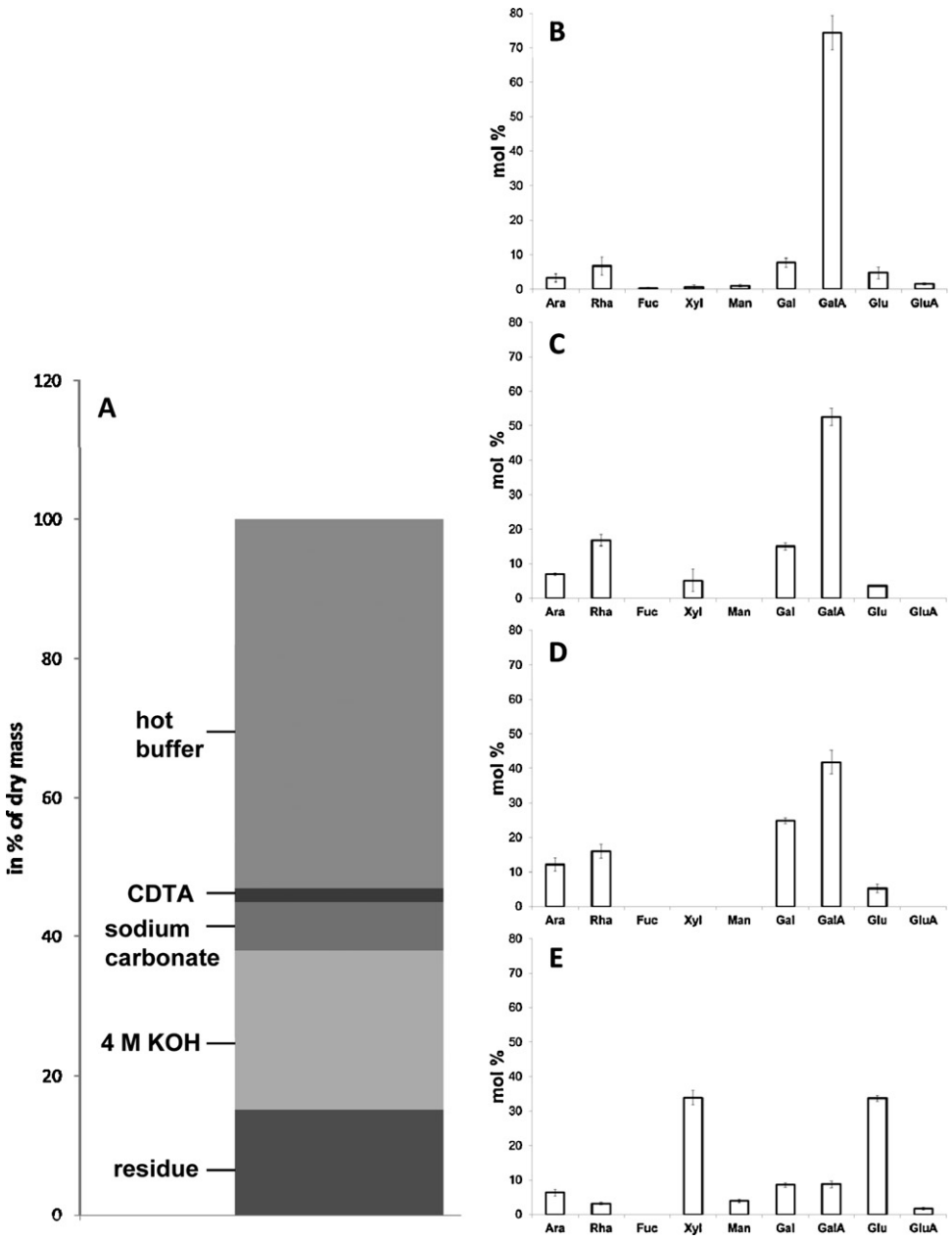


**Fig. 3.** Gravimetric analysis and monosaccharide composition of various chemically-extracted fractions from alcohol insoluble residue from mature tobacco leaves (for the fractionation process see A). Gravimetric analysis is shown in (A) and monosaccharide composition of the corresponding chemically-extracted fractions in (B–E). (B) 100 °C sodium acetate buffered extract. (C) CDTA extract, (D) $Na_2CO_3$ extract and E: 4 M KOH soluble extract. Ara, arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid.

cell wall composition suggests that substituted RG-I and RG-II pectins are not major pectin components in tobacco. *Arabidopsis* leaves were also shown to display a similar cell wall monosaccharide composition to these tobacco data (Nguema-Ona et al., 2006; Caffall et al., 2009). In *Arabidopsis*, Zablackis et al. (1995) estimated that 34% of the total wall polysaccharides were solubilised in phosphate buffer and that this fraction is generally not included when evaluating the total wall polymer composition (or AIR; Zablackis et al., 1995). The contribution of these buffer soluble pectic polysaccharides is also, as in the case of *Arabidopsis*, not reflected in the compositional data reported here for tobacco.

Following on from this initial analysis, a chemical (Fig. 2A) as well as an enzymatic (Fig. 2B) fractionation scheme was used, in parallel, on the AIR in order to provide further insight into the wall polymer networks of the tobacco leaf tissue. Gravimetric analysis (Fig. 3A) of the chemically extracted fractions obtained from the AIR revealed that ca. 50% (w/w) of extracted material consisted of hot buffer soluble material, whereas ca. 2% (w/w) was extracted further using the divalent ion chelator 2-diaminocyclohexanetetraacetic acid (CDTA) and a further 6% (w/w) could be obtained by sodium carbonate treatment. Addition of 4 M KOH extracted an additional 20% (w/w) of materials with the remaining residue comprising ca. 16% (w/w) of the total material. Although Zablackis et al. (1995) used a different scheme to fractionate *Arabidopsis* AIR, their gravimetric analysis showed that *Arabidopsis* leaves (AIR fraction) contained approximately 42% of pectin (23% HG, 11% RG-I and 8% RG-II) and 24% hemicelluloses (20% XyG and 4% xylan). When

compared to our data (an average of 50-55% HG, 4-10% RG-I and RG-II, 20% hemicelluloses), it is clear that tobacco fully expanded leaves contain more pectin and slightly less hemicellulose.

In order to ascertain the main wall polymers present in each of these fractions, monosaccharide compositional analysis was performed (Fig. 3B–E). The hot buffer soluble material (Fig. 3B) comprised primarily (ca. 75–80 mol%) of GalA indicating that 60% (w/w) of the total leaf cell wall was pectin, of which the bulk occurs in the form of HG chains (see also Eda and Kato, 1980). The 1% (w/w) CDTA-extractable fraction (Fig. 3C) consisted of ca. 50 mol% GalA, 20 mol% Rha and 15 mol% Gal, indicating a more branched pectin structure present, probably consisting of HG chains, RG-I and possibly RG-II polymers. Sodium carbonate extraction, which is known to cleave ester linkages, resulted in a fraction containing ca. 40 mol% GalA, 25 mol% Gal, 15 mol% Rha and 10 mol% Ara (Fig. 3D). This indicated that branched RG-I structures with galactan and arabinan chains that contain ester linkages are present. These linkages were not extractable using aqueous buffers or chelating agents and suggest that branched RG-I compounds from this fraction could be entangled with, or directly attached to other wall polymer systems (e.g. hemicelluloses) (Fry, 2011). Strong alkali treatment (4 M KOH) produced a fraction (Fig. 3E) containing mainly Xyl and Glc (at ca. 35 mol% each) indicating that the bulk of this fraction consisted of AXyGs (Eda and Kato, 1978; Mori et al., 1979) and glucuronoxylan polymers (Eda et al., 1977). The low levels of Ara present support the previous data indicating that *N. tabacum* hemicellulose comprises AXyGs – further detailed structural analyses are presented later in this section. The low levels, generally less than 10 mol%, of Rha, Man, Gal and GalA (Fig. 3E) present in this fraction indicated that tightly bound pectin (e.g. RG-I) and mannans are probably also present. Eda et al. (1982) also noticed the presence of RG-I pectic polysaccharides in this alkaline fraction. The remaining residue was found to consist mainly of Glc (data not shown) and is therefore inferred to be primarily cellulose which is known to be insoluble in the extractants used.

Fourier-transform infrared (FT-IR) spectroscopy was used to provide further support to our inferences regarding the presence of specific wall polymers in the different fractions (Fig. 4). Spectral analysis of total AIR revealed spectral maxima at 1630 $cm^{-1}$ and at 1017 $cm^{-1}$, as well as bands between 1200 and 1300 $cm^{-1}$ (Fig. 4A). This tobacco AIR spectrum is similar to that obtained for *Arabidopsis* dark-grown hypocotyls (Mouille et al., 2003). From literature (Kacurakova et al., 2000) it is known that the main maxima at 1017 $cm^{-1}$ indicates HG presence and the minor band at ca. 1400 $cm^{-1}$ correlates to the associated carboxylate functional group. This reinforces the previous compositional data (Figs. 1 and 3) indicating that pectin is the most abundant polymer of *N. tabacum* leaf cell walls. Additional bands at ca. 1600 $cm^{-1}$ and 1350–1250 $cm^{-1}$ correlate to proteins (i.e. amide groups) and lipids (i.e. hydrocarbon chains) respectively. Chemical fractionation of the AIR (Fig. 2A) allowed us to probe the pectic and hemicellulosic polymers of the wall network using FT-IR spectroscopy (Fig. 4B–E). The hot buffer fraction displays a spectrum (Fig. 4B) with a major maxima at 1017 $cm^{-1}$ and local maxima at 1580 $cm^{-1}$ and 1740 $cm^{-1}$. The major maxima correlated to HG chains whereas the local maxima most likely represented a combination of amide (e.g. water soluble proteins) and ester absorbances (Kacurakova et al., 2000). Further extraction of the pectic material using CDTA showed major absorbance bands at 1580 $cm^{-1}$ and 1380 $cm^{-1}$ (Fig. 4C). These bands were interpreted to be due to residual CDTA in the fraction which was difficult to remove even after extensive washing and dialysis. Identical bands were also found in the sodium carbonate extracted material (Fig. 4D) indicating residual CDTA presence. As the CDTA absorbs in a region of the IR spectrum where protein groups and not wall carbohydrate polymers absorb, it was not considered to significantly influence the data analysis or the

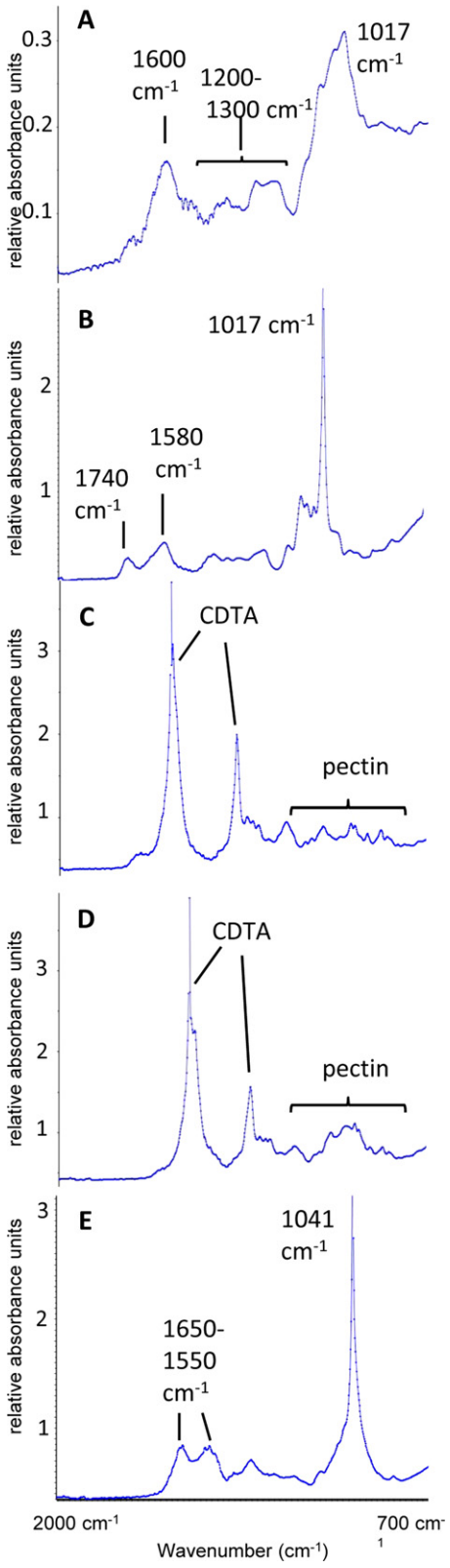


**Fig. 4.** Fourier transform infrared (FT-IR) spectra generated from alcohol insoluble residue prepared from tobacco leaves and associated chemical fractions (for the fractionation process see Fig. 2A). (A) total alcohol insoluble residue, (B) 100 °C sodium acetate buffered extract, (C) CDTA extract, (D) $Na_2CO_3$ extract and (E) 4 M KOH extract.

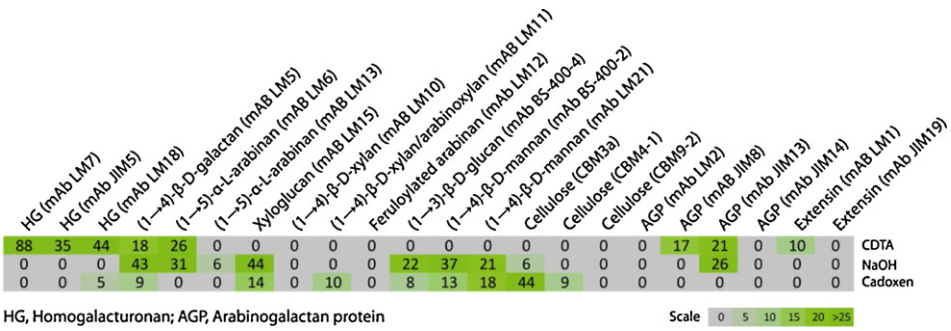


**Fig. 5.** CoMPP (comprehensive microarray polymer profiling) analysis of tobacco leaf cell wall fractions. The heatmap shows the relative abundance of plant cell-wall glycan-associated epitopes present in tobacco leaf cell wall material and colour intensity is correlated to mean spot signals. Sequential extractions were carried out with CDTA (top row), NaOH (middle row) and cadoxen (bottom row) and the extracted material spotted onto nitrocellulose which was probed with sets of antibodies and carbohydrate binding modules (see Table 1 for antibody codes and information). The values in the heatmap are mean spot signals from three experiments and the highest signal in the entire data set was set to 100 and all other data adjusted accordingly. A cut off value of 5 was imposed.

interpretation. However, the gravimetric analysis (Fig. 3A) probably overestimates the contribution of the extractable material using CDTA and sodium carbonate to the total polymer content. This is evident from inspecting the CDTA spectrum (Fig. 4C) which shows no significant absorbance in the wall carbohydrate rich region 1200–900 $cm^{-1}$. The sodium carbonate IR spectrum (Fig. 4D) however shows a band around 1060–1000 $cm^{-1}$, supporting the chemical fractionation data that indicated some branched pectic material to be liberated, possibly through the covalent cleavage of ester bonds due to weak alkali (i.e. carbonate ions). Treatment with KOH yielded a major wall fraction which showed a main maximum at 1041 $cm^{-1}$ and two local maxima at ca. 1650 $cm^{-1}$ and 1550 $cm^{-1}$ (Fig. 4E). The sharp absorbance peak at 1041 $cm^{-1}$ correlated to XyG absorption (Kacurakova et al., 2000) and concurred with the monosaccharide compositional data reported (Fig. 3E). The local maxima (Fig. 4E) correlated to amide bands (e.g. protein) and hence appeared to represent wall-bound proteins which require strong alkali to liberate them, probably via a combination of hydrogen bond disruption and covalent cleavage processes. Finally, the remaining residue was found to consist primarily of crystalline and amorphous cellulose (data not shown) which is not extractable with the solvents and solutions used, again confirming the chemical fractionation data.

To complement these chemical and spectroscopic analyses and results, and to provide additional independent data, CoMPP analysis of chemical fractions of AIR was performed. CoMPP analysis allows the profiling and the analysis of cell wall material using sets of monoclonal antibodies (mAbs) and carbohydrate binding modules (CBMs) which show specificities toward plant cell wall-glycan epitopes (Moller et al., 2007). CoMPP analysis provides information about the relative abundance of epitopes in the extracted material rather than fully quantitative data but in contrast to fully quantitative biochemical techniques, it can provide information about polysaccharide rather than monosaccharide occurrence. The CoMPP data (Fig. 5 and Table 1) strongly supported the chemical and spectroscopic data reported. The use of three extractions in the CoMPP analysis is comparable to the five fractions obtained using chemical fractionation. The CDTA extract should be similar to the combination of the hot buffer and CDTA extracts performed previously for gravimetric and compositional analysis. The NaOH extracted material should be a combination of the sodium carbonate and KOH fractions whereas the cadoxen (cadmium oxide with diaminoethane) extract probably represents some of the insoluble residue obtained at the end of the chemical fractionation scheme. Analysis of the CDTA extract (Fig. 5A) showed that the HG epitopes recognised by mAbs JIM5, JIM7 and LM18 are present and confirms that HG polymers occur in the AIR. The binding of mAbs LM5 and LM6 (Fig. 5A), indicating the presence of galactan and arabinan epitopes respectively, confirms the presence of the branched pectin domains RG-I. Additionally, arabinogalactan proteins (AGPs) appear also to be extracted by the CDTA extract, as shown by the binding of mAbs JIM8 and JIM13 (Fig. 5A). The binding of mAb LM15 to the NaOH fraction supports the presented data regarding the XyG-rich nature of the strong alkali extractable material (Fig. 3D). Additional epitopes recognised by anti-mannan mAbs LM21 and BS-400-2 and the anti-(1 → 3)-β-glucan mab BS-400-4 suggest the extract is rich in hemicellulosic polymers (Fig. 5B). Co-extracted pectic- and AGP-containing material appear also to be present, given the binding of mAbs LM5, LM6, LM18 and JIM13 to the NaOH extracted material (Fig. 5B). Like NaOH, cadoxen is a strong base which is effective at solubilising hemicelluloses and also partially solubilises cellulose. As expected, XyG was present in the cadoxen fraction, as was residual (1 → 3)-β-glucan, mannan, galactan and a low level of HG. The cellulose epitope recognised by CBM3a was present in the cadoxen fraction, and the xylan epitope recognised by mAb LM11 was also detected.

In addition to the chemical fractionation, enzymatic fractionation was also performed (see Fig. 2B). This approach involved the use of highly specific enzyme preparations to 'dissect' the wall polymer networks from each other (Persson et al., 2011). The fractionation scheme involved initial sequential digestion with endopolygalacturonase (EPG)-containing preparations, followed by dividing the de-pectinated residue into two samples and then comparing the effect of two different XEG enzymes with each other on this material (Fig. 2B). In parallel, the 4 M KOH soluble fraction was also used to compare the efficacy of both XEGs. Monosaccharide compositional analysis of the fractions obtained from EPG digestion in buffered sodium acetate yielded GalA (ca. 50 mol%), Gal (ca. 20 mol%), Rha (ca. 10 mol%) and Ara (ca. 5 mol%) (Fig. 6A). This again suggested a pectin-rich extract composed of both HG chains and branched structures (e.g. RG-I). Further treatment of the residue from the first digestion with EPG supplemented with CDTA resulted in release of additional pectic material (Fig. 6B) which had a similar monosaccharide composition (Fig. 6B) as the first extract (Fig. 6A). However, the second extract had more branched pectin, indicated by the slight shift in the overall molar ratio toward Ara and Rha and away from Gal and GalA. FT-IR spectroscopy of both EPG liberated fractions confirmed the pectic nature with strong absorbance maxima in both spectra at 1017 $cm^{-1}$ (data not shown).

The de-pectinated residue (hence enriched for hemicelluloses and cellulose) and the 4 M KOH soluble fraction were then used as starting material to extract and analyse the AXyG structure of *N.*

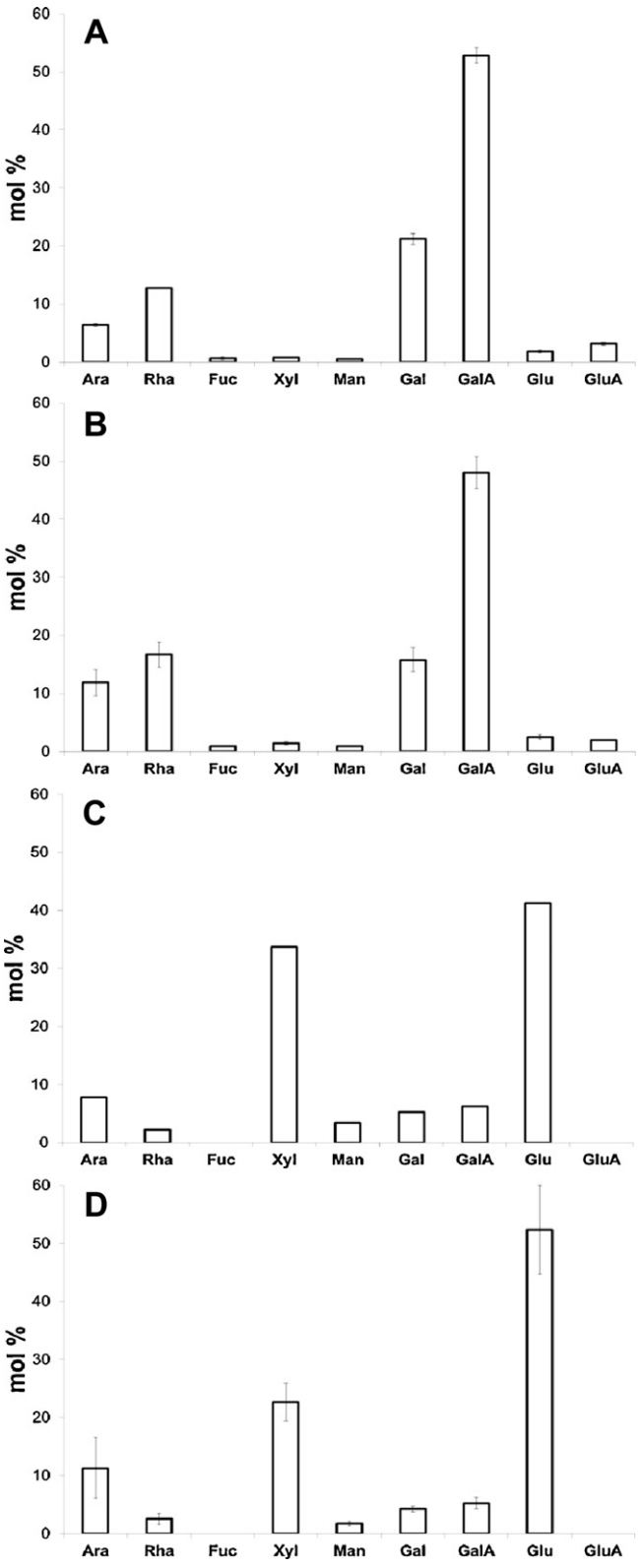


**Fig. 6.** Monosaccharide compositional analysis of EPG-mediated and XEG-mediated soluble fractions of alcohol insoluble residue prepared from tobacco leaves (for the fractionation process see Fig. 2A). (A) Fraction resulting after EPG digestion of previously saponified alcohol insoluble residue. (B) Fraction resulting after EPG digestion in the presence of a chelating agent (CDTA) of the previous residue. (C) Fraction resulting after *Tr*EGII digestion (see Fig. 3E). (D) Fraction resulting after *Psp*XEG5 digestion of the 4 M KOH soluble fraction (see Fig. 3E).

*tabacum* leaf cell walls. The efficacy and specificity of two commercially available XEGs were tested. *Tr*EGII (XEG74; Grishutin et al., 2004) and *Psp*XEG5 (XEG5; Yaoi et al., 2005) differ in their molecular structures and modes of action. *Tr*EGII belongs to the glycosyl hydrolase (GH) family 74 (www.cazy.org) and contains two main domains: a catalytic domain and a cellulose binding domain with a mode of action similar to exo-type enzymes (Grishutin et al., 2004). In contrast, *Psp*XEG5 belongs to the GH5 family and was the first XEG described in this family (Yaoi et al., 2005). *Psp*XEG5 does not have a cellulose binding domain and is described as a true endo-type enzyme. *Psp*XEG5 has not been tested thus far against XXGG-type xyloglucan found predominantly in Solanaceae. Digestion of the de-pectinated residue (data not shown) and the 4 M KOH soluble fraction with the *Tr*EGII yielded as the main monosaccharides Xyl and Glc at ca. 30 mol% and 40 mol%, respectively (Fig. 6C). Lesser amounts, ca. 5–10 mol% of Ara, Rha, Gal and GalA were also present in the digestion extract (Fig. 6C). These data strongly suggest that the extract is rich in XyGs, however the unusually high amount of Xyl also present implies that xylans comprise a significant component of the extract. This is not unexpected given that the endoglucanase preparation used is known to possess low levels of xylanase activity (www.megazyme.com). By comparison, use of the *Psp*XEG5 preparation results in a much simpler chromatograph (Fig. 6D), showing Glc (ca. 50 mol%), Xyl (ca. 20 mol%) and Ara (ca. 10 mol%) as the main monosaccharides (Fig. 6D). Additional sugars present comprise less than 10 mol% of the total molar composition (Fig. 6D). The 1:2:5 molar ratio for Ara:Xyl:Glc observed is identical to that obtained from previous studies on arabinoxyloglucan polysaccharides isolated from *N. plumbaginifolia* suspension cultured cells (Sims et al., 1996).

In order to more fully investigate the compositional and structural complexity of the arabinoxyloglucan polymers present, and to compare the specificity of the two XEGs available towards tobacco AXyGs, a combination of chromatographic and mass spectrometric (i.e. ESI-MS) analysis was performed on the digestion products obtained from the two enzyme treatments. The analyses were conducted on de-pectinated AIR residue (hence enriched for hemicellulose and cellulose).

Firstly, linkage analyses of both extracts showed that they predominantly consist of AXyG with the major peak being a 4-Glc (Table 2 and Fig. 7B and C), instead of 2- and 4-linked Xyl when the hemicellulose fraction is analysed (Table 2 and Fig. 7A). Additional PMAA derivatives which eluted here (Fig. 7A–C) confirmed the hemicellulose-rich (Fig. 7A) and the AXyG-rich (Fig. 7B and C) composition of the analysed materials (see also Eda and Kato, 1978; Mori et al., 1979). 2-Linked (present in AXyG) and 4-linked-xylosyl derived PMAAs (present in glucuronoxylan; Eda et al., 1977) co-elute in several gas liquid chromatography columns (Carpita and Shea, 1989) including the RTX 5 SIL-MS column (this study). We performed methylation analysis of a purified mixture of tamarind xyloglucan oligosaccharides and β-1,4 xylotetraose (both from Megazyme) which confirmed co-elution at 19.8 min (data not shown). The occurence of these two xylosyl derived PMAAs can be used to ascertain the specificity of the commercial XEGs which are known to often exhibit additional or contaminating β-1,4 glycanase activities including β-1,4 xylanase and β-1,4 mannanase activities (www.cazy.org; www.megazyme.com). To compare the specificity of the two XEGs in this study, we examined the fragmentation pattern of the PMAA derivatives eluting at 19.8 min. We show that the 4 M KOH soluble fraction and the *Tr*EGII-soluble fraction contain both 2-linked- and 4-linked-xylosyl derived PMAAs with contrasting abundance. The 4-linked xylosyl residue is more abundant in the 4 M KOH soluble fraction (Fig. 7D) while the 2-linked xylosyl residue is more abundant in the *Tr*EGII-soluble fraction (Fig. 7E), confirming the XEG activity of the latter. The *Psp*XEG5-soluble fraction contains only a 2-linked xylosyl derived PMAA (Fig. 7F) rather

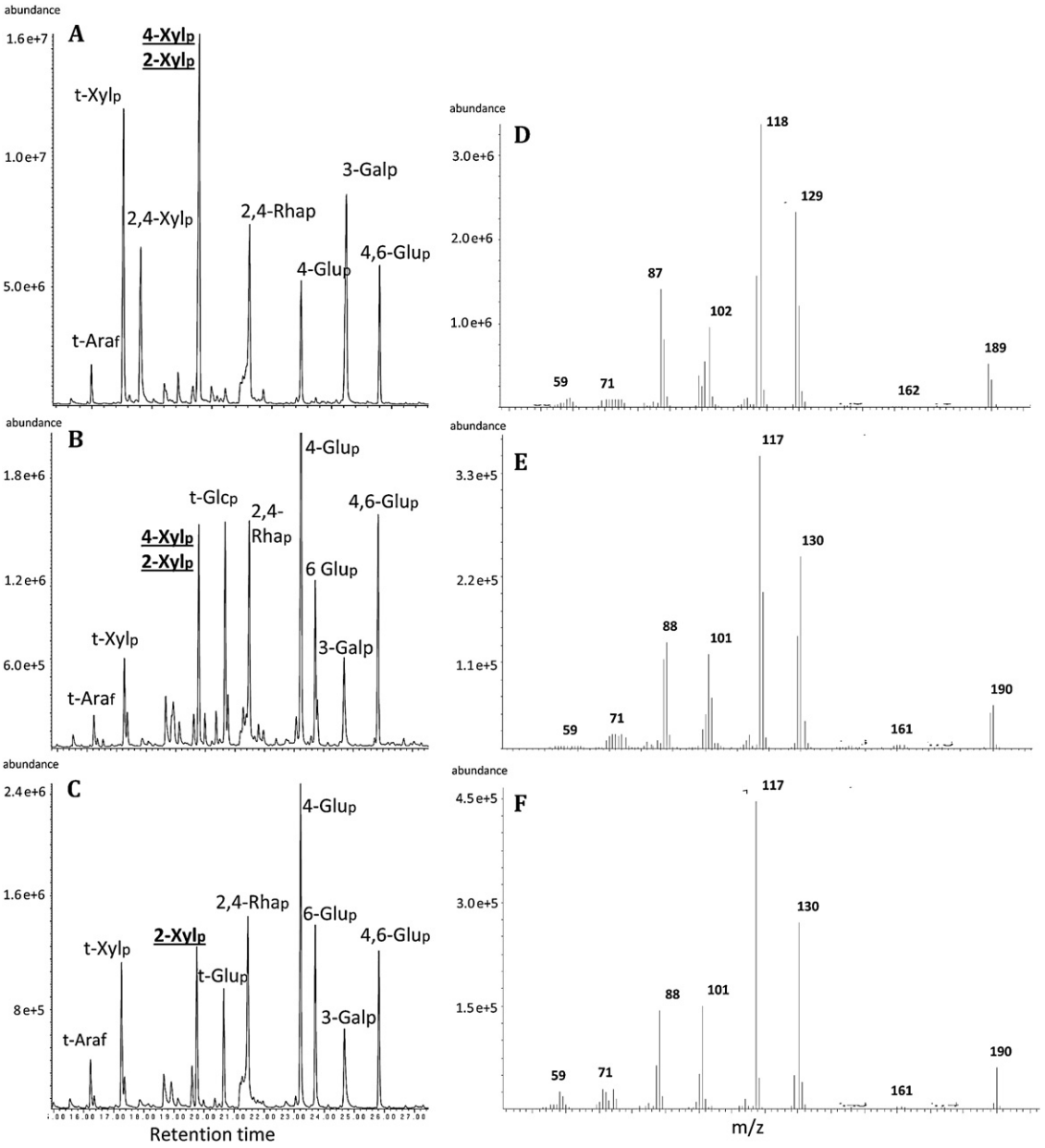


**Fig. 7.** Linkage analysis of tobacco hemicellulose-enriched (4 M KOH soluble) fraction and XEG-soluble fraction from leaves. (A–C) TIC (total ion chromatogram) of the hemicellulosic fraction (A), the *Tr*EGII-suloble fraction (B) and the *Psp*XEG5-soluble fraction (C). D-F: spectra showing the fragmentation pattern of PMAAs derivatives eluting approximately at 19.8 min in the hemicellulose fraction (D), the *Tr*EGII-suloble fraction (E) and the *Psp*XEG5-soluble fraction (F). Note that the hemicellulose fraction (A and D) and *Tr*EGII-soluble fraction (B and E) show fragmentation patterns overlapping both 2-linked- and 4-linked-xylosyl PMAA derivatives at 19.8 min. In contrast, *Psp*XEG5-soluble fraction shows a fragmentation pattern characteristic of a 2-linked xylosyl PMAA derivative being the only species present at 19.8 min.

than a mixture with 4-linked-xylosyl derivatives, confirming the high specificity of the enzyme toward AXyGs.

Secondly, HPAEC analysis of the *Tr*EGII extract yielded a complex mixture of oligomeric fragments consisting of at least eleven separate oligosaccharides (Fig. 8A). In contrast, *Psp*XEG5 digestion yielded a simpler chromatographic trace displaying at least nine oligosaccharides (Fig. 8B). The additional oligosaccharides in the *Tr*EGII digestion, when compared to the *Psp*XEG5 treatment, may result from the endo-processive mode of action of the enzyme toward XyG, rendering the unsubstituted glucose residues of the backbone polymer digestible by *Tr*EGII (Grishutin et al., 2004). In the case of tobacco XXGG-type XyG which have less substituted glucose residues, this may result in the release of additional oligosaccharides. Additional oligosaccharides may also result from the contaminating xylanase and mannanase activities and thus could consist of xylan- or glucomannan-derived oligomers. ESI-MS analysis provided further detailed structural information on the components present in both *Tr*EGII and *Psp*XEG5 solubilised extracts (Fig. 8C and D). The main ions present in both digests (Fig. 8C and D) at *m*/*z* 791, 923, 1085, 1248 and 1409 correlated to $Hex_3Pen_2$, $Hex_3Pen_3$, $Hex_4Pen_3$, $Hex_5Pen_3$ and $Hex_6Pen_3$ oligomers respectively and are confirming data obtained for *N. plumbaginifolia* suspension cultured cells (Sims et al., 1996). Furthermore, no major differences were observed between ESI-MS spectra of *Tr*EGII-versus *Psp*XEG5-mediated digestion (Fig. 8C and D).

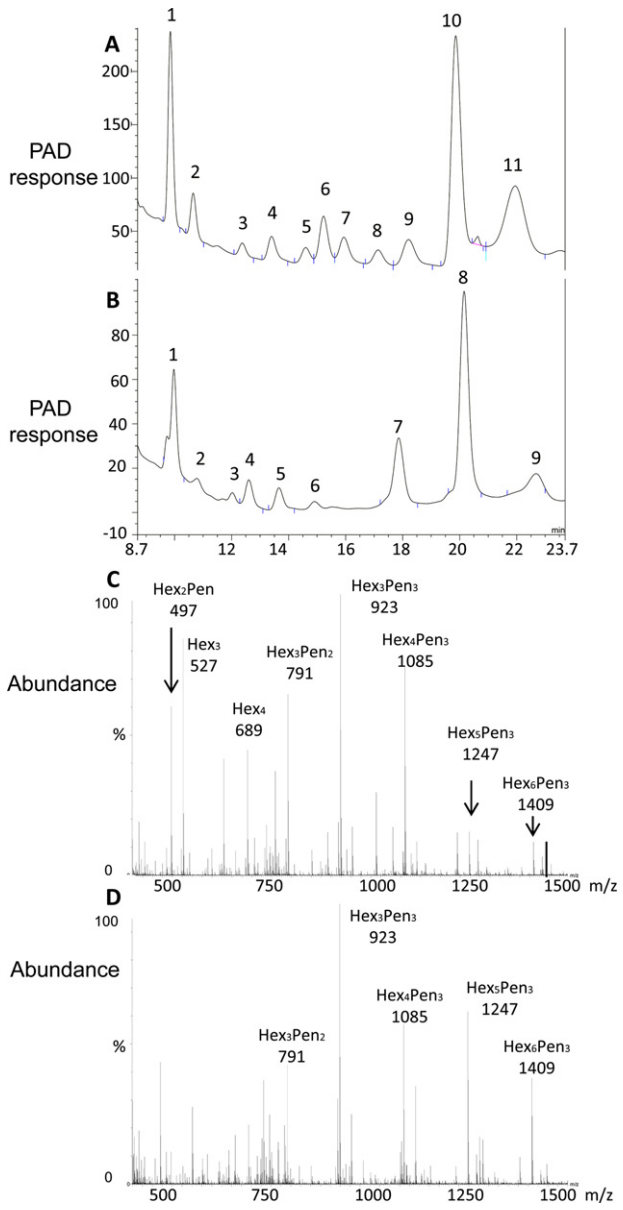


**Fig. 8.** A comparison of oligosaccharides obtained from tobacco arabinoxyloglucans (enriched from de-pectinated alcohol insoluble residue) using *Trichoderma reseei* endoglucanase II (*Tr*EGII) or *Paenibacillus* sp. XEG5 (*Psp*XEG5) enzyme treatment followed by (a) high performance anion exchange chromatography (A and C *Tr*EGII) and (b) ESI mass spectrometry (B and D *Psp*XEG5).

## 4. Conclusion

Tobacco remains an important plant model system and there is an associated need for the development and/or implementation of state-of-the art tools to analyze tobacco plants, including their cell walls. Tobacco plants are used, among other species, as model plants to perform functional gene studies, such as antifungal research on grapevine pathogens performed in our laboratory (laboratory of MAV, JPM, E-NO). The lack of validated cell wall profile methods and reference data on mature tobacco tissue is a hindrance to progress in studying transgenic *N. tabacum* populations generated for this research. Although previous studies, using mostly classical polysaccharide isolation and carbohydrate chemical analysis methods applied to tobacco suspension cells, have already provided important insights into the pectin-rich cell walls of tobacco, this species has not yet benefited from the cell wall profiling methods that have been mainly optimized for the model plant, *Arabidopsis thaliana*. A range of techniques have now been performed on tobacco samples and the data obtained provides a basis for a proposed guideline of procedures and methods to analyze and profile the pectin-rich cell walls of tobacco leaves. It was also noted that during sample preparation for tobacco leaves (i.e. pre-processing to AIR) that: (1) the leaves have a thick protective cutin layer that limits accessibility to extractants/enzymes, (2) the high lipid content prevents efficient sample manipulation and (3) in the case of arabinoxyloglucan analysis the pectin-rich material blocks endoglucanase action. Thus any direct analysis of tobacco leaves proved to be highly problematic and unsatisfactory, hence all analysis was performed on AIR. Monosaccharide compositional analysis and FT-IR spectroscopy provide rapidly obtainable datasets albeit the information obtainable is limited to the broad composition of the walls present. Similarly, CoMPP analysis is useful in a screening context but provides additional specificity by highlighting polymers present, via their epitopes, although the data is semi-quantitative in nature. Clearly as a profiling technology, CoMPP analysis provides probably the most informative set of data but requires the use of complementary compositional analysis techniques if a more detailed analysis of wall structural composition (or associated changes) is required. A powerful approach, in a screening context, would be to combine these three types of data using multivariate methods to rapidly evaluate mutant and transgenic tobacco populations for changes in wall structure and composition. From the tobacco data reported here it was evident that the detailed chemical fractionation protocol employed in combination with FT-IR spectroscopic and monosaccharide compositional analysis was useful to define appropriate extraction protocols as, for example, it was clear that a single hot buffer extract was sufficient for liberating the majority of the pectins and that subsequent chelator addition was superfluous. Finally, an enzymatic oligosaccharide fingerprint approach was developed for analyzing the specific AXyG polymers present in the leaves. Enzymatic digestion of the AIR coupled to chromatographic separation and mass spectrometric analysis of oligosaccharides generated, as well as monosaccharide compositional analysis of the associated fractions, proved a powerful approach to probe for more detailed structural information. The *Psp*XEG5F enzyme was found to be more suitable for profiling the specific XXGG-type of XyGs present in tobacco as it appeared to exclusively release AXyG oligosaccharides. This needs further confirmation, particularly as not all of the oligosaccharides released are yet identified, and more detailed analyses are necessary to elucidate the mechanism(s) associated with the XXGG-specific activity of this enzyme. However, in a screening context the less specific mode of action of the *Tr*EGII enzyme may be advantageous, as this could allow the simultaneous evaluation of oligosaccharides generated from AXyGs, xylans and mannans present in tobacco tissue rather than performing three separate chemical analyses.

It is evident from these analyses that each of the profiling approaches that were implemented provided complementary datasets (i.e. gravimetric and compositional, spectroscopic, immunological, chromatographic and mass spectrometric data) on the structural composition of tobacco leaf cell walls. Each profiling technique has distinct advantages and disadvantages, and while it is clear that the compositional and spectroscopic methods are more relevant in a screening context it is also clear that more detailed datasets are obtainable using a fractionation protocol coupled to enzymatic fingerprinting. Due to the lack of tobacco mutants or associated genetic resources available a thorough validation of our procedure is not currently possible. However, a comparison of this set of data with similar datasets generated with *A. thaliana* leaves (albeit under different conditions which could account for some of this variation) reveals differences in profiles arising from CoMPP

analysis (Moller et al., 2007), XyG enzymatic oligosaccharide fingerprinting (Lerouxel et al., 2002) and compositional studies (Zablackis et al., 1995). We believe this emphasizes the fact that reference cell wall datasets are not easily transferable across species and that not only is it necessary to provide reference data for *Nicotiana* species (as now is available for *N. tabacum* from this study), but also for others model crop plant species, such as tomato (*S. lycopersicum*), potato (*S. tuberosum*) and grapevine (*Vitis vinifera*).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2012.01.044.

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