Carbohydrate Polymers 88 (2012) 939–949

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

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

Eric Nguema-Ona[a], John P. Moore[a,*], Alexandra Fagerström[b], Jonatan U. Fangel[b], William G.T. Willats[b], Annatjie Hugo[c], Melané A. Vivier[a]

[a] Institute for Wine Biotechnology, Department of Viticulture and Oenology, Faculty of AgriSciences, Stellenbosch University, Matieland 7602, South Africa
[b] Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, DK-1001, Denmark
[c] Department of Microbiology, Stellenbosch University, Matieland 7602, South Africa

ARTICLE INFO

Article history:
Received 14 September 2011
Received in revised form 13 January 2012
Accepted 16 January 2012
Available online 1 February 2012

Keywords:
Profiling
Tobacco leaves
Cell wall polysaccharides
Pectin-rich
Endopolygalacturonase (EPG)
Arabinoxyloglucan
Xyloglucan-specific endoglucanase (XEG)

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.

* Corresponding author. Tel.: +27 21 808 2733; fax: +27 21 808 3771.
E-mail address: moorejp@sun.ac.za (J.P. Moore).

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doi:10.1016/j.carbpol.2012.01.044

& 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