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Short communication

Competitive binding of pectin and xyloglucan with primary cell wall cellulose

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ABSTRACT

Assemblies of pectin, xyloglucan and cellulose were studied *in vitro* using two ternary systems. In the first one, xyloglucan concentration varied, while pectin amount was kept constant. In the second one, pectin concentration varied, whereas xyloglucan amount was fixed. The use of ternary systems allowed to put forward the hypothesis that pectin/cellulose and xyloglucan/cellulose associations may exist together or separately, depending on the proportion of non-cellulosic polysaccharides in cell walls. It can be hypothesized that pectin plays a double role within primary cell walls: (i) pectin loosely bound to cellulose, in xyloglucan-rich cell walls, (ii) pectin associated with cellulose, in xyloglucan-poor cell walls.

1. Introduction

Primary cell walls of dicotyledons might be considered as a mixture of biopolymers, essentially polysaccharides, among which cellulose, xyloglucan and pectin are of peculiar importance. Cellulose is synthesized as a linear chain of β-(1→4)-linked Glc*p* residues. Parallel glucan chains are associated by hydrogen bonds to form microfibrils. Xyloglucan, a major hemicellulose in primary cell walls, has a cellulose-like backbone branched at *O*-6 by α-D-Xyl*p* residues. Some of Xyl*p* residues can be substituted at *O*-2 by Gal*p* residues, which can be further branched at *O*-2 by Fuc*p* residues. Pectin constitutes a highly complex and heterogeneous group of polysaccharides, composed of distinctive covalently linked domains. Two main structural domains can be distinguished: homogalacturonan (HG) and rhamnogalacturonan I (RG I) (O'Neill, Albersheim, & Darvill, 1990). HG is a homopolymer composed of α-(1→4)-linked GalA*p* residues, whereas RG I contains a backbone of the repeating disaccharide unit: (1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*. Primary cell wall pectins are characterised by a high quantity of neutral sugar side chains, mostly composed of Ara and Gal that are mainly branched at *O*-4 of Rha residues (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

Cellulose and xyloglucan are known to form a network mediated most likely by non-covalent linkages, whereas pectin is thought to fill the interstices within the cellulose/xyloglucan network (Carpita & Gibeaut, 1993). Pectin, that is believed to form an independent network, plays the role of plasticizer and water-binding agent. The primary cell wall model proposed by Carpita and Gibeaut (1993) is based on the assumption that primary cell walls contain an equal amount of cellulose and xyloglucan. However, the proportion of cellulose, xyloglucan and pectin varies among different plant species. High amounts of pectin and cellulose, with very low xyloglucan content, were reported in many primary cell walls of mature plants, e.g., sugar beet (Renard & Jarvis, 1999), potato (Zykwinska, Ralet, Garnier, & Thibault, 2005), celery (Thimm et al., 2002), onion (Hediger, Emsley, & Fischer, 1999) and carrot (Stevens & Selvendran, 1984). On the opposite, only few cell walls contain important amounts of xyloglucan, e.g. pea (Hayashi & Maclachlan, 1984) and blackberries (Hilz, Bakx, Schols, & Voragen, 2005).

Several *in vitro* studies provided evidence that xyloglucan can coat and/or tether cellulose microfibrils through non-covalent interactions (Hayashi, Marsden, & Delmer, 1987; Pauly, Albersheim, Darvill, & York, 1999; Vincken, Keizer, Beldman, & Voragen, 1995; Whitney, Brigham, Darke, Reid, & Gidley, 1995). Similar artificial composites created with pectins or isolated pectic domains have shown that pectins can also bind to cellulose microfibrils through their neutral sugar side chains (Zykwinska et al., 2007a, 2005). The pectin/cellulose interactions were also suggested by other authors (Iwai, Ishii, & Satoh, 2001; Oechslin, Lutz, & Amado, 2003; Vignon, Heux, Malainine, & Mahrouz, 2004). Indeed, in the cell walls of *Nicotiana plumbaginifolia*, Iwai et al. (2001) evidenced that arabinose-rich pectins are strongly associated with cellulose-hemicellulose complexes. Oechslin et al. (2003) suggested

Abbreviation: CWM, cell wall material.

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interactions between cellulose and pectic galactan side chains in apple cell walls. Moreover, an arabinan-cellulose "composite" was isolated from the spine fibers of the cactus *Opuntia ficus-indica* after successive alkali extractions (Vignon et al., 2004).

In the present work, we studied the hypothesis if the two major primary cell wall networks, namely xyloglucan/cellulose and pectin/cellulose exist together or separately within cell walls. For this purpose, an *in vitro* approach was developed and two pectin/xyloglucan/cellulose ternary model systems were created. The results were compared to the ones obtained in the binary *in vitro* pectin/cellulose and xyloglucan/cellulose systems. Polysaccharides representative of primary cell walls: (i) arabinan-rich sugar beet pectin, (ii) fucosylated pea xyloglucan and (iii) primary cell wall cellulose were used.

2. Materials and methods

2.1. Substrates

Sugar beet pectin was extracted from sugar beet cell wall material (CWM) (Cagny sugar factory) in mild alkaline conditions (0.05 N NaOH, 4 °C, 3 × 30 min), as previously described by Zykwinska et al. (2005).

Xyloglucan was extracted from pea pods CWM. Pea pods (from local market) were cut onto small pieces and left for 12 h with 75% (v/v) ethanol (5 L). The slurry was filtered through a nylon cloth. The insoluble material was left for another 12 h with 75% (v/v) ethanol (5 L) and filtered again. This step was carried out four times. The residue was then dried by solvent exchange (ethanol, acetone) and left overnight at 40 °C. Pea pods CWM (5 g) were stirred with 400 mL of 4 M KOH and 26 mM $NaBH_4$ at 40 °C for 16 h. After filtration through a G3 sintered glass, the supernatant was neutralized with 1 M CH_3COOH, dialyzed against distilled water and freeze-dried. Purified pea xyloglucan was recovered after anion-exchange chromatography performed on a DEAE Sepharose CL-6B column (30 × 2.6 cm) (Pharmacia). A solution of 40 mL of the extract (~200 mg) was loaded onto the column equilibrated with 50 mM Na–succinate buffer (pH 4.5) at a flow rate of 1.5 mL/min. The column was first eluted with 370 mL of 50 mM Na–succinate buffer (pH 4.5). A NaCl gradient (0–0.6 M NaCl) was then applied. Fractions of 15 mL were collected and analyzed colorimetrically for their content in total neutral sugars and GalA. Purified xyloglucan was eluted with 50 mM Na–succinate buffer (pH 4.5) and 0 M NaCl (unbound fraction).

The primary cell wall cellulose was prepared from sugar beet CWM as described by Heux, Dinand, and Vignon (1999) and Zykwinska et al. (2005). Briefly, sugar beet CWM was sequentially treated with hot dilute acid (0.1 M HCl, 85 °C, 3 × 30 min) and hot dilute alkali (0.5 M NaOH, 80 °C, 3 × 30 min) in order to solubilize pectins and hemicelluloses. The primary cell wall cellulose thereby obtained was suspended in distilled water, mixed in a Warring Blender and homogenized by 10 passes through an APV Gaulin homogenizer operating at 1000 bars.

2.2. Binding assays

2.2.1. Binary xyloglucan/cellulose and pectin/cellulose systems

Binding assays in binary systems were performed as described elsewhere (Zykwinska et al., 2005). Briefly, solutions of xyloglucan or pectin were prepared at different concentrations (C_0 = 2.5–1000 μg/mL) and aliquots (V) (1.5 mL) were mixed with a known amount of cellulose (m) (~5 mg). After incubation under continuous head-over-tail mixing at 40 °C for 6 h, the blends were centrifuged, and the supernatants were tested for their total neutral sugar contents by colorimetric assays. The amount of adsorbed matter per mg of cellulose (q_e) was calculated from the difference in sugar content measured for xyloglucan or pectin solutions (C_0) and blends supernatants (C_e), taking into account the amount of sugars released in the blank (cellulose in Na–acetate buffer, pH 5.8), with the following equation:

$$q_e = \frac{(C_0 - C_e)V}{m} \quad (1)$$

Binding assays were performed in triplicate. The average and the corresponding error of measurement were calculated for each point.

2.2.2. Ternary pectin/xyloglucan/cellulose systems

Solutions of xyloglucan or pectin were prepared at 1 mg/mL in 20 mM Na–acetate buffer (pH 5.8) and eventually diluted to give a range of concentrations: $C_0 \approx 50$, 100, 200, 500, 1000 μg/mL. Additionally, solutions of pectin or xyloglucan were prepared at $C_0 \approx 500$ μg/mL. After centrifugation for 15 min at 4000*g*, xyloglucan/pectin mixtures were prepared (1.5 mL of each aliquot of diluted xyloglucan (or pectin) was mixed with 1.5 mL of pectin (or xyloglucan) aliquot at 500 μg/mL). Xyloglucan/pectin mixtures were then added to cellulose (~5 mg). Polysaccharide solutions and polysaccharide/cellulose blends incubated 6 h at 40 °C (head-over-tail mixing) were centrifuged for 15 min at 9000*g*, and supernatants (1250 μL) were removed for analysis. Neutral sugar contents were quantified in polysaccharide solutions and blends supernatants, as their alditol acetate derivatives. The amount of adsorbed matter was calculated from the difference in sugar contents measured for pectic solutions and blends supernatants, taking into account the amount of sugars released in the blank (cellulose in Na–acetate buffer, pH 5.8). The amount of xyloglucan adsorbed onto cellulose was estimated according to Glc residues. The amount of arabinan-rich pectin adsorbed onto cellulose was calculated according to Ara residues, after taking into account the amount of Ara residues in pea xyloglucan samples.

Binding assays were performed in triplicate. The average and the corresponding error of measurements were calculated for each point.

2.3. Analytical

Uronic acid (as GalA) and total neutral sugars contents were determined colorimetrically by the automated *m*-hydroxybiphenyl and orcinol methods, respectively (Thibault, 1979; Tollier & Robin, 1979).

The individual neutral sugars were analyzed as their alditol acetate derivatives by gas liquid-chromatography (Blakeney, Harris, Henry, & Stone, 1983) after hydrolysis by 2 M H_2SO_4 at 100 °C for 2 h for pea xyloglucan and sugar beet pectin. Supernatants removed after binding assays were hydrolyzed by 2 M TFA at 120 °C for 2 h. Primary cell wall cellulose was first prehydrolyzed by 72% (w/v) H_2SO_4 for 1h30 at 25 °C, then hydrolyzed by 2 M H_2SO_4 at 100 °C for 2 h. Inositol was used as an internal standard.

The molar mass distribution and polydispersity index were determined by light scattering detection after high performance size exclusion chromatography. The system used was composed of one Shodex SB-G pre-column followed by two Shodex OH-pak SB HQ 805 and 804 columns in series with a multiangle laser light scattering detector (MALLS; mini Dawn, Wyatt, Santa Barbara, CA, USA), a differential refractometer (ERC 7517 A) and a differential viscometer (T-50A, Viscotek). Elution was performed with 50 mM $NaNO_3$ containing 0.02% NaN_3 at a flow rate of 0.7 mL/min at room temperature. The system was calibrated using pullulan standards.

3. Results

Binding assays using pea xyloglucan and sugar beet pectin were performed under *in vitro* conditions. Both polysaccharides, representative of primary cell walls, were tested for their ability to interact with primary cell wall cellulose. The adsorption of xyloglucan and pectin onto cellulose in ternary pectin/xyloglucan/cellulose systems was therefore compared to that measured in binary xyloglucan/cellulose and pectin/cellulose ones.

3.1. Polysaccharides composition and characteristics

The sugar composition and physico-chemical characteristics of the polysaccharides used in the present study are presented in Table 1. Pea xyloglucan is a fucogalactoxyloglucan, with a molar ratio Fuc:Gal:Xyl:Glc of 1:3:5:10, close to that determined for apple xyloglucan (1:1.5:4:6) by Renard, Voragen, Thibault, and Pilnik (1991). Sugar beet pectin is particularly rich in arabinan side chains, which constitute ~20% of the pectin weight (Table 1). The degree of methylation of pectin extracted in mild alkaline conditions was around 16 (Zykwinska et al., 2005). It appeared that xyloglucan and pectin have similar weight-average molar masses M_W, estimated at ~143 kDa and ~98 kDa for xyloglucan and pectin, respectively. The polydispersity index I determined was higher than 1 (Table 1), which highlights an important heterogeneity of both non-cellulosic polysaccharides. Primary cell wall cellulose extracted from sugar beet cell walls is mainly composed of Glc residues, which constitutes ~92% of the cellulose weight. Cellulose preparation did not lead to total extraction of pectic and hemicellulosic sugars, which represented around 7% of the cellulose weight (Table 1). Chemical extraction of matrix polysaccharides provoked the collapse of cellulose microfibrils and a mechanical treatment leading to their separation was necessary. The applied treatment allowed to obtain a stable mixture of individual and bundled cellulose microfibrils of apparent random orientation (Fig. 1). Based on X-ray diffraction results previously published (Zykwinska et al., 2007a), primary cell wall cellulose extracted from sugar beet cell walls is rather amorphous and contains around 40% of ordered zones. The degree of crystallinity corresponds well to the results published by Heux et al. (1999). It was previously proposed (Heux et al., 1999; Wormald, Wickholm, Larsson, & Iversen, 1996) that the mechanical process applied to cellulose may lead to conversion between disordered and ordered zones. This observation suggests that some constrained parts of the microfibrils, appearing as disordered zones, can be cut during the process, which increases the apparent crystallinity index. Therefore, chemical and mechanical treatments applied to extract and defibrillate cell walls cellulose may alter its native structure.

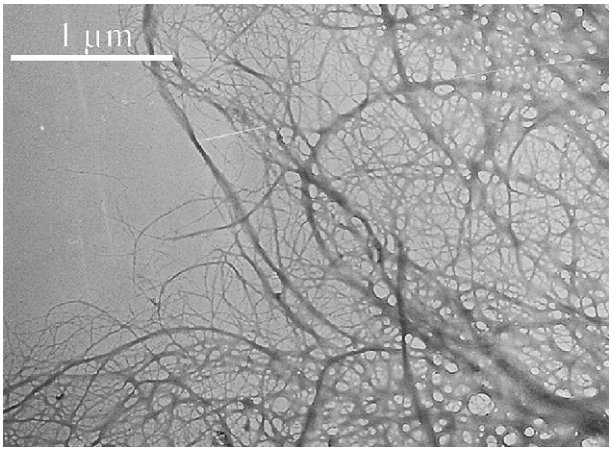

Fig. 1. TEM micrograph of the primary cell wall cellulose.

3.2. Binding isotherms

3.2.1. Xyloglucan/cellulose binary and xyloglucan/pectin/cellulose ternary systems

The binding isotherms of fucosylated xyloglucan adsorbed onto cellulose in the binary xyloglucan/cellulose and ternary xyloglucan/pectin/cellulose *in vitro* systems are presented in Fig. 2. The amount of adsorbed xyloglucan onto cellulose in the binary system increased with increasing concentration and no apparent plateau-value was observed in the range of concentrations used. A maximum binding of ~112 µg of xyloglucan per mg of cellulose was measured at 1000 µg/mL of xyloglucan (Fig. 2). Using similar substrates (pea xyloglucan and amorphous cellulose), Hayashi, Ogawa, and Mitsuishi (1994) observed a maximum adsorption of 167 µg of xyloglucan per mg of cellulose. In a previous study (Zykwinska et al., 2005), a maximum plateau-value of only 33 µg of xyloglucan per mg of cellulose, reached above 750 µg/mL of xyloglucan, was observed for non-fucosylated tamarind xyloglucan adsorbed onto primary cell wall cellulose.

In the ternary system, the concentration (C_0) of xyloglucan varied from 25 to 500 µg/mL, while the amount of pectin was kept constant at 250 µg/mL. When looking at the adsorption of xyloglucan, it appears that the amount of xyloglucan adsorbed onto cellulose in the presence of pectin (ternary system) was very close to that observed in the absence of pectin (binary system), for initial xyloglucan concentrations from 25 to 250 µg/mL. More differences were observed for the highest xyloglucan concentration at 500 µg/mL (corresponding to $C_e \approx 250$ µg/mL, Fig. 2). This better xyloglucan binding onto cellulose surface could be explained by a local increase of xyloglucan concentration, which might be due to a phase separation phenomenon. It can be hypothesized that at high polysaccharides concentration, pectin and xyloglucan are not mixed in a homogeneous manner but form micro domains that are more or less enriched in one of them. Therefore, a micro domain enriched in xyloglucan located near cellulose microfibrils may explain better adsorption compared to a binary system, where xyloglucan is more likely distributed in a homogeneous way in the whole sample volume.

When looking at the adsorption of pectin (at 250 µg/mL) in the presence of different xyloglucan concentrations (from 25 to 500 µg/mL), it can be noticed that the amount of pectin adsorbed

Table 1
Sugar composition and macromolecular characteristics of primary cell wall cellulose (PCW cellulose), pea xyloglucan and sugar beet pectin

	Neutral sugars and galacturonic acid (mg/g)								Physico-chemical parameters	
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	M_w(kDa)	$I(M_w/M_n)$
PCW cellulose*	0	0	2	26	18	4	925	17	nd	nd
Pea xyloglucan	0	42	31	202	6	152	463	0	143	2.7
Sugar beet pectin*	26	2	201	2	1	55	4	589	98	5.4

M_W – weight-average molar mass; I – polydispersity index.
* Data from Zykwinska et al. (2005).

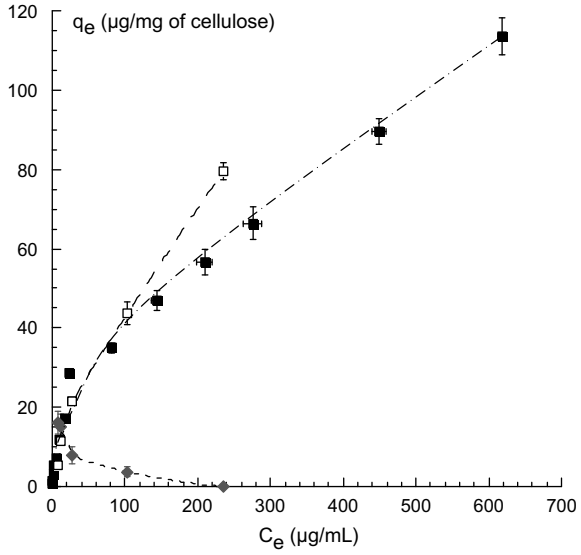

Fig. 2. Binding isotherms of fucosylated pea xyloglucan onto cellulose in the binary xyloglucan/cellulose system (■) and in the ternary xyloglucan/pectin/cellulose system (□), where q_e, the amount of polysaccharide adsorbed per mg of cellulose; C_e, the concentration of free polysaccharide remaining in solution after adsorption step. In the ternary system, xyloglucan concentration was varied (25, 50, 100, 250, 500 µg/mL), while the pectin concentration was kept constant (~250 µg/mL) (♦ – the amount of pectin (µg/mg of cellulose) adsorbed in parallel to xyloglucan for each xyloglucan concentration used).

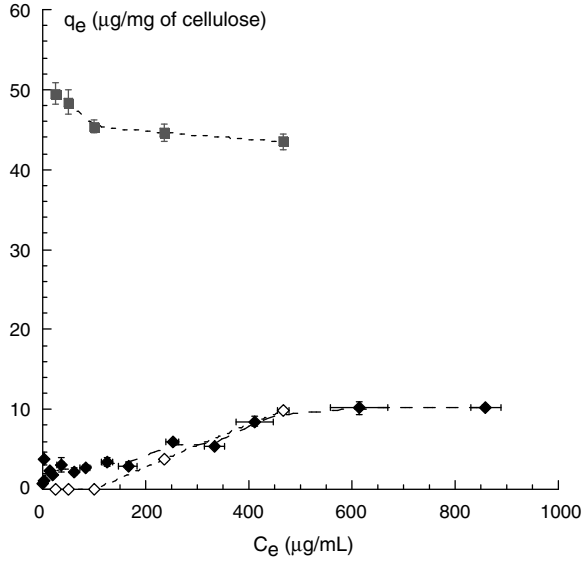

Fig. 3. Binding isotherms of arabinan-rich sugar beet pectin onto cellulose in binary pectin/cellulose system (♦; Zykwinska et al., 2005) and in ternary xyloglucan/pectin/cellulose system (◇). In the ternary system, pectin concentration was varied (25, 50, 100, 250, 500 µg/mL), while the xyloglucan concentration was kept constant (~250 µg/mL) (■ – the amount of xyloglucan (µg/mg of cellulose) adsorbed in parallel to pectin for each pectin concentration used).

onto cellulose decreases, when the concentration of xyloglucan increases (Fig. 2). At low concentration of xyloglucan (25 and 50 µg/mL), ~16 µg of pectin was adsorbed per mg of cellulose, while at high xyloglucan concentration (500 µg/mL), no pectin was bound onto cellulose.

3.2.2. Pectin/cellulose binary and pectin/xyloglucan/cellulose ternary systems

The binding isotherms of the arabinan-rich pectin adsorbed onto cellulose in the binary and ternary *in vitro* systems are presented in Fig. 3. In the binary pectin/cellulose system, the amount of pectin adsorbed onto cellulose increased with increasing concentration and a plateau value of ~8 µg/mg of cellulose was reached above 500 µg/mL of pectin (Zykwinska et al., 2005).

In the ternary system, the pectin concentration varied from 25 to 500 µg/mL, while the xyloglucan concentration was kept constant at 250 µg/mL. In the presence of xyloglucan (ternary system), no binding of pectin onto cellulose was observed for low pectin concentrations from 25 to 100 µg/mL (Fig. 3). However, increasing pectin concentration (>100 µg/mL) led to a pectin adsorption similar to that observed in the binary pectin/cellulose system. When looking at the adsorption of xyloglucan, it can be noticed that the amount of xyloglucan bound decreases slightly, when the concentration of pectin increases (Fig. 3). Cellulose microfibrils adsorbed ~49 µg of xyloglucan per mg of cellulose in the presence of 25 µg/mL of pectin, while ~43 µg of xyloglucan per mg of cellulose was bound onto cellulose in the presence of 500 µg/mL of pectin (Fig. 3).

4. Discussion

In the present work, the binding ability of xyloglucan and pectin to cellulose in ternary pectin/xyloglucan/cellulose systems was studied in comparison to the adsorption observed in binary xyloglucan/cellulose and pectin/cellulose ones. Fucosylated pea xyloglucan, sugar beet pectin rich in arabinan side chains and cellulose, representative of higher plants primary cell walls, were used. Sugar beet pectin extracted in mild alkaline conditions has a lower degree of methylation (16) than native pectin (50–60). It can however be thought that the degree of methylation of pectin will not affect pectin binding to cellulose in the conditions used, since it was previously established that binding is governed by pectic neutral sugar side chains and not by pectic backbone (Zykwinska et al., 2007a, 2005; Zykwinska, Thibault, & Ralet, 2007b).

The binding behaviour of xyloglucan to cellulose does not seem affected by the presence of pectin, as the isotherms obtained for xyloglucan in the presence of pectin (ternary system) and in the absence of pectin (binary system), were very similar. It is unclear why, at low xyloglucan concentration, the arabinan-rich pectin displayed a higher adsorption onto cellulose (~2 times) in comparison to the adsorption of pectin alone onto cellulose in the binary pectin/cellulose system. Although the hypothesis of covalent interaction between pectin and xyloglucan was already put forward (Popper & Fry, 2005; Thompson & Fry, 2000), the possibility of covalent bonding between these two entities in our *in vitro* systems is very unlikely. Moreover, it was observed that when the amount of xyloglucan adsorbed onto cellulose surface increases, the pectin adsorption is prevented. This result suggests that pectin/cellulose interaction is weaker than the interaction between xyloglucan and cellulose. Similar observations were made in the second ternary system, where an adsorption of pectin onto cellulose was measured in the presence of xyloglucan. No binding of pectin onto cellulose was observed at low pectin concentrations, whereas xyloglucan exhibited the same binding behaviour as in the binary xyloglucan/cellulose system. Increasing pectin concentration resulted in an increased adsorption of pectin onto cellulose similar to that observed in the binary pectin/cellulose system.

In the present study, the adsorption of xyloglucan and pectin onto cellulose in artificial *in vitro* systems allowed to evidence that these polysaccharides are able to bind to cellulose surface simultaneously. However, it appeared that xyloglucan and pectin are most likely in competition to form associations with cellulose. Indeed, when the proportion of xyloglucan is high compared to pectin, very low pectin adsorption is then measured. On the opposite, when the

concentration of xyloglucan is low, the pectin adsorption is relatively high. These findings suggest that pectin may perform a double role within the primary cell wall. It can be hypothesized that in the presence of high amounts of xyloglucan, pectin is loosely attached to cellulose and fills the interstices within xyloglucan/cellulose network, while xyloglucan interacting with cellulose microfibrils is mainly responsible for maintaining the cell wall architecture. On the other hand, in cell walls that are poor in xyloglucan (sugar beet, potato, celery, onion, carrot), pectin, that is particularly abundant in those cell walls, could replace xyloglucan in binding the gaps between microfibrils and maintaining their separation. Indeed, it was recently demonstrated that single pectin and/or pectin network are able to tether cellulose microfibrils through pectic neutral sugar side chains (Zykwinska et al., 2007b).

Other ternary *in vitro* composites (pectin/xyloglucan/cellulose) were previously studied by Chanliaud, Burrows, Jeronimidis, and Gidley (2002). In those composites, cellulose was synthesized by *Acetobacter xylinus* in a medium containing tamarind xyloglucan and apple pectin. On average, the ternary composites consisted of 63% cellulose, 22% pectin and 15% xyloglucan, compared to 39% pectin or 22% xyloglucan incorporated in binary pectin/cellulose or xyloglucan/cellulose composites (Chanliaud et al., 2002). These results highlight that in ternary composites incorporation, not only of pectin but also of xyloglucan, in the freshly synthesized cellulose, was affected. This observation confirms our hypothesis assuming that pectin and xyloglucan are in competition to form associations with cellulose microfibrils.

It is believed that cell wall expansion occurs when the hydrogen bonds between xyloglucans and cellulose are broken or removed by expansins (McQueen-Mason & Cosgrove, 1994). Additionally, xyloglucan endotransglucosylase/hydrolase (XTHs) participate in wall loosening by cutting the xyloglucan tethers between cellulose microfibrils and introducing new xyloglucan fragments (Fry et al., 1992). By analogy with Fantner et al. (2005), it can be hypothesized that during cell wall growth, weak bonds between pectic side chains and cellulose microfibrils are broken (sacrificed) and the wall stretches out. The stretching absorbs energy and prevents the wall from breaking. After this step, the broken "sacrificial" bonds are reformed, which restores the wall strength and resilience.

The *in vitro* findings that cellulose can bind simultaneously xyloglucan and pectins may allow to enlarge our knowledge of the primary cell wall architecture. The hypothesis that primary cell walls are composed of at least three interacting networks: xyloglucan/cellulose, pectin/cellulose and proteins can be put forward.

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