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Fine structure of a mixed-oligomer storage xyloglucan from seeds of *Hymenaea courbaril*

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Abstract

Seed storage xyloglucans of *Hymenaea courbaril* possess a structure composed of xylocellopentaosyl (XXXXG) and xylocellohexaosyl (XXXXXG) backbone units in addition to the more common xylocellotetraosyl (XXXG) units. Electrospray mass spectrometry confirmed that both types of units exist in the same polymer. The storage xyloglucan gives a xylocellotetraose:xylocellopentaose:xylocellohexaose ratio of about 2:1:0.2. Incomplete digestion of xyloglucan gave dimers of these units in ratios of tetramer–tetramer (T-T), tetramer–pentamer (T-P), and pentamer–pentamer (P-P) backunits of 3.1:2.1:1.0, far from the 4:4:1 ratio expected if the assembly were random. Although discovery of the xylocellopentaosyl units requires a re-evaluation of the cellobiosyl/cellotetraosyl unit mechanism of backbone synthesis, a 4–5–5–4 unit ordered assembly that accounts for the dimer ratios observed preserves a mechanism by which the even-number cellodextrin units are added. Three distinct, singly galactosylated oligomers from the xylocellopentaosyl units were isolated and characterized, with XXXLG being the predominant oligomer of this type in the polymer. Examination of galactosylation profiles of the dimers indicates that the position of these subtending residues is also not random.

Keywords: *Hymenaea courbaril*; Cell wall; Xyloglucan; Storage polysaccharides; Electrospray ionization-mass spectrometry

1. Introduction

Xyloglucan (XyG) is a major matrix glycan in the primary walls of all dicots and about one-half of all monocot species (Carpita & Gibeaut, 1993). Some seeds store this polysaccharide in large amounts as a resource for the embryo after germination (Buckeridge, Santos, & Tiné, 2000). Typically, XyGs comprise $(1 \rightarrow 4)$-β-D-glucan backbones, with three of every four glucose residues substituted with $(1 \rightarrow 6)$-α-D-xylopyranosides to give the fundamental xylocellotetraosyl unit structure, XXXG.[2] This basic structure was suggested many years ago from the proportions of

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Abbreviations used: ESI-MS, electrospray ionization-mass spectrometry; Glc, glucose; H, (galacto)xylocellohexaosyl units; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; P, (galacto)xylocellopentaosyl oligomeric units; T, (galacto)xylocellotetraosyl oligomeric units; XyG, xyloglucan; XET, xyloglucan endotransglucosylase (enzyme activity); XTH, xyloglucan endotransglucosylase/hydrolase (gene/protein family).

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[2] A single letter code representing the non-reducing terminal residue of branch points along the main chain has been adopted for xyloglucan structures (Fry et al., 1993), with G representing an unbranched D-glucose residue, X representing a D-xylosyl residue attached to the O-6 of the Glc, and L representing a D-galactosyl residue attached to the O-2 of the subtending xylosyl residue. The (galacto)xylocellodextrin oligomers from xyloglucan are generated by action of the *Trichoderma endo*-β-glucanohydrolase that cleaves the backbone only at unbranched glucosyl residues.

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monosaccharides and methylation analysis (Kooiman, 1960). The most widespread assay for XyG fine structure is by digestion with a *Trichoderma endo*-β-glucanohydrolase (*endo*-β-glucanase) and separation of the oligomers by high performance anion-exchange liquid chromatography. This enzyme hydrolyses XyG polymers only at unbranched glucosyl residues (Anderson & Stone, 1975; Kooiman, 1961). This action usually produces several structurally distinct oligosaccharides from the XXXG structure, but differing from each other by extension of the xylosyl side-chains with one or two galactose units, and further with fucose (Buckeridge, Rocha, Reid, & Dietrich, 1992; Vincken, Beldman, & Voragen, 1997).

Xyloglucans are synthesized at the Golgi membrane by nucleotide-sugar requiring enzymes (Faik, Desveaux, & Maclachlan, 1997; Gordon & Maclachlan, 1989; Hayashi & Matsuda, 1981). Because XyG and cellulose share the same glycosyl backbone, cellobiose has been proposed as the basic unit of synthesis of both polysaccharides (Faik et al., 1997; Farkas & Maclachlan, 1988; Hayashi & Matsuda, 1981), a restriction imposed by the inversion of the glycosyl residues typical of $(1 \rightarrow 4)$-β-linkages (Carpita & Vergara, 1998; Carpita, McCann, & Griffing, 1996). One of the few exceptions to the XXXG-based XyG is the polysaccharide found in Solanaceae, where only two contiguous glucose residues are substituted with xylose (York, Kolli, Orlando, Albersheim, & Darvill, 1996). But even in this instance, the third glucosyl residue is acetylated at the O-6, and the basic structure of cellotetraosyl backbone (XXGG) units is maintained.

When storage XyG from *Hymenaea courbaril* L. was digested with the *Trichoderma endo*-β-glucanase, (galacto) xylocellopentameric (P) and even small amounts of (galacto)xylocellohexameric (H) backbone oligosaccharides were observed in addition to the usual family of xylocellotetrameric (T) backbones oligomers (Buckeridge et al., 1997). The first oligosaccharide of this series to be purified and characterized was XXXXG, but these authors pointed out other unidentified oligosaccharides produced by the *Trichoderma endo*-β-glucanase from XyG and suggested the presence of a family of galactosylated oligosaccharides with five glucoses in the main chain and with xylocellotetramer:xylocellopentamer:xylocellohexamer units in a ratio of 2:1:0.15.

In this work, we show by electrospray ionization mass spectrometry that fragments with two constitutive blocks of the *H. courbaril* XyG contain both series of oligosaccharides (XXXG-, XXXXG-, and XXXXXG-based) in the same polymer. A stochastic, or random, arrangement of a 2:1 ratio of xylocellotetraosyl and xylocellopentaosyl units would give a ratio of dimer combinations of 4:4:1 (T-T:T-P/P-T:P-P). Surprisingly, the dimers had a ratio close to 3:2:1, which indicates a deviation from random distribution. Of several models of block-wise arrangements we tested to account for this ratio, only a model of synthesis that places P-P dimers sandwiched in any order between a twofold excess of xylocellotetraosyl units consistently gives such a ratio. Synthesis of xylocellopentaosyl units produced in pairs in a T-P-P-T arrangement, i.e. 18 consecutive glucosyl residues within the XyG synthase, requires a much larger synthase machinery than previously anticipated.

2. Materials and methods

2.1. Collection of fruits of *H. courbaril*

Fruits of *H. courbaril* were collected from a mature tree at the gardens of the Institute of Botany at São Paulo, Brazil. The collection began in May (Autumn), five months after flowering, and continued up to September (Spring). Length and width of the fruits were recorded, and the fruits were then dissected to obtain the seeds. The seeds were dried and pulverized in a ball mill, and the XyGs were extracted from these dry powders as described below. As flowering was observed to be highly variable, we found that the use of the ratio length:width of the fruit was a more suitable marker of storage deposition instead of anthesis.

2.2. Extraction of xyloglucans

XyGs were prepared from the powder of seeds of *H. courbaril* as described by Buckeridge et al. (1997), modified from Kooiman (1961). Briefly, XyGs were extracted from the dry powders in distilled water at 80 °C for 8–9 h. After filtration through a nylon mesh to remove insoluble residues, soluble XyGs in the supernatant were precipitated upon addition of three volumes of ethanol, then incubated for 15 h at 5 °C. The precipitated XyGs were washed with acetone, air-dried and re-dissolved in distilled water at room temperature. The solution was centrifuged at 12,000*g* for 10 min, and this operation was repeated until no debris were found as precipitated material. The material was deionized by passing the solution through a mixed-bed resins column of Dowex 50 W × 8, 50–100 mesh (cation) and Dowex 1 × 8, 50–100 mesh (anion) (Sigma, St. Louis, MO) and eluted with distilled water. The solution was freeze-dried and stored at room temperature.

To generate oligosaccharides, 5 mL of a 0.5% (w/v) solution of XyG in 50 mM sodium acetate, pH 5.0, and was hydrolyzed after addition of 200 μL of a 1:100 dilution in water of *Trichoderma viride* cellulase (500 U mL^{-1} in 3.2 M ammonium sulfate, pH 7; Megazyme, Wicklow, Ireland) for up to 24 h at 30 °C. The limit-digest oligosaccharides were separated from a small amount of undigested polysaccharide by precipitation with 3 vol of ethanol. The supernatant was freeze-dried and submitted to HPAEC-PAD analysis as described below.

2.3. Purification of the oligosaccharides

For the production of high molecular weight fragments of xyloglucan, solutions of storage xyloglucans (0.1 or

1.0%, w/v) were prepared on 50 mM Na-acetate buffer, pH 5.0. Two hundred microlitres of the polysaccharide solution were incubated with 50 μL of cellulase (1.0 U mL^{-1}). For assays with lower concentration of cellulase, the solution of cellulase used was 0.5 U mL^{-1}. The solution of oligosaccharides produced was loaded onto a column of Biogel P-6 (Bio-Rad, Richmond, CA) and eluted with water containing 0.05% sodium azide. Fractions (1 mL) were collected, and carbohydrates were quantified by the phenol–sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). High performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was with a CarboPac PA-100 column (Dionex, Sunnyvale, CA, USA) with pulsed amperometric detection (HPAEC-PAD; Dionex, Sunnyvale, CA, USA). The separation was performed in 88 mM NaOH at 0.9 mL min^{-1} with a linear gradient of sodium acetate in 88 mM NaOH from 35 to 75 mM for 20 min, and then from 75 to 200 mM for 30 min. Fractions that contained the same oligosaccharide were pooled and deionized with Dowex columns as described above.

The procedure was based on the separation of the cellulase limit digest xyloglucan oligosaccharides in a long (1.5-cm diameter × 170-cm long) column of Biogel P-6. After analysis by HPAEC-PAD of 1-mL fractions, we found that although having the same molecular mass, the three oligosaccharides appeared in different fractions in a highly purified form. Samples of limit digest oligosaccharides were chromatographed through the Biogel P-6 column several times until sufficient amounts of the three oligosaccharides were obtained for MS/MS analyses.

2.4. Monosaccharide analysis

Volumes equivalent to 50 μg of each purified oligosaccharide were freeze-dried in glass vials and dissolved in 350 μL of M HCl. The tubes were sealed and heated at 100 °C for 2 h. The monosaccharides produced were analyzed by HPAEC-PAD in a CarboPac PA-1 column (Dionex, Sunnyvale, CA, USA) isocratically in 20 mM NaOH at 0.8 mL min^{-1}.

2.5. Isolation of β-galactosidase from cotyledons of *Copaifera langsdorffii* and digestion of *H. courbaril* xyloglucan oligomers

Seeds of *C. langsdorffii* were collected from trees cultivated at the Institute of Botany of São Paulo (Brazil) and stored at 5 °C. Seeds were soaked in water, and after full imbibition, were planted in pots containing a mixture of forest soil and sand (1:1; v/v). The β-galactosidase was purified to homogeneity as described by Alcântara, Dietrich, and Buckeridge (1999) from cotyledons at stage 9, as previously described in this work. The collection of cotyledons by morphological stage instead of time was used because of the high degree of variability of seed development. No residual activities of either α-xylosidase or β-glucosidase were present in the β-galactosidase preparation, as judged by HPAEC analysis of the products of hydrolysis of a mixture of XyG oligosaccharides. The β-galactosidase activity of the final preparation, as judged by release of Gal from *p*-nitrophenol-β-galactoside, was 23.3 μmol Gal min^{-1} mg $protein^{-1}$ at 30 °C.

To XyG oligomers in 100 μL portions of the fractions obtained from Biogel P-6 chromatography were added 20 μL of β-galactosidase preparation, and the mixture was incubated at 30 °C for 24 h. Oligomers generated were assayed by HPAEC upon direct loading of the digestion reactions.

2.6. Linkage analysis

The freeze-dried oligomers stored over P_2O_5 in a vacuum desiccator were methylated with *n*-butyllithium and methyl iodide as described in Gibeaut and Carpita (1991). Partly methylated alditol acetates were separated by gas–liquid chromatography and identified by electron-impact mass spectrometry as described by Carpita and Shea (1989).

2.7. Electrospray MS and MS/MS

The samples were analyzed by electrospray MS on a Finnigan MAT liquid chromatograph mass spectrometer (Thermoquest Corp., San Jose, CA). Freeze-dried samples containing 0.1–1 mg of the oligomer preparations were dissolved in 100 μL of water, and 10 μL of glacial acetic acid and 10 μL of methanol were added. The source voltage was set at 3.5 kV, the capillary voltage was varied between 20 and 30 V, depending on sample abundance, and the capillary temperature was 225 °C. Typical background source pressures were 1.5×10^{-5} torr as indicated by an ion gauge. The sample flow rate was 10 μL min^{-1}, and the drying gas was nitrogen. The samples were scanned from m/z 300 to m/z 2000 for analysis of monomer units and from m/z 2000 to m/z 3500 for dimer units. For MS/MS experiments, helium was introduced into the system to an estimated pressure of 1×10^{-3} torr to improve trapping efficiency, and it also acted as the collision gas during collisionally activated decomposition of selected trapped ions. The collision energy was set at 40–50% of a maximum of 5 V "tickle" voltage, depending on abundance and mass of the fragment.

3. Results and discussion

3.1. Characterization of storage xyloglucan oligomers

The sizes of the seeds of *H. courbaril* vary considerably, and a better measure of developmental maturity is the length:width ratio (Fig. 1A). The storage XyG is deposited in the walls of the cotyledons at the end of the maturation period of the seed during early July. During a cell expansion phase when primary wall XyG deposition ensues,

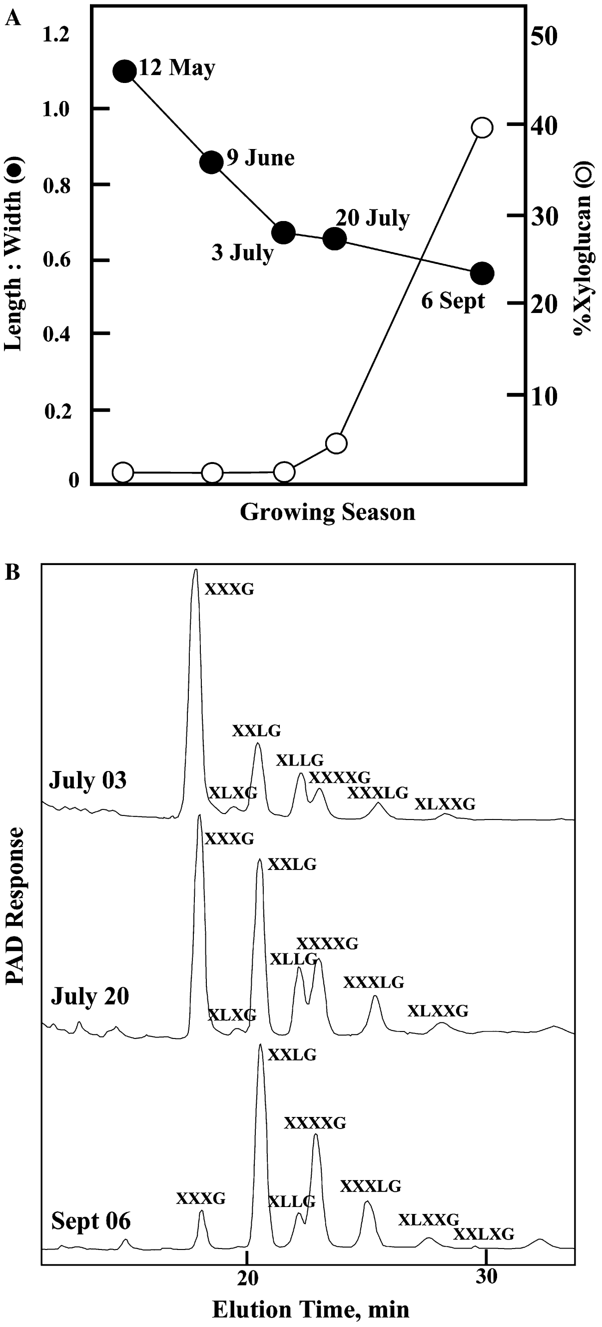

Fig. 1. Accumulation and changing oligomeric profiles of hot water-extractable XyGs during development and maturation of the seed. (A) Maturation of the seed expressed as an increase in width relative to length. Cotyledons reached their maximum size about July 3, after which XyGs accumulate to nearly 40% (w/w) of dry mass. (B) Analysis by HPAEC-PAD of the oligosaccharides from *Hymenaea courbaril* storage XyGs isolated at various stages of seed development and early maturation and hydrolyzed with *endo*-β-glucanase. Before deposition, the polymer is composed mainly of XXXG, whereas afterwards the storage XyGs are composed of high amounts of XXLG and XXXXG-based oligosaccharides.

the polymer consists of primarily xylocellotetraose units onto which both galactosyl and fucosyl residues are added. However, when storage XyG deposition commences, the biosynthetic mechanism shifts to synthesize galactosylated xylocellopentaosyl units as well (Fig. 1B). Although the polysaccharide can be modified after deposition on the wall, in muro modifications of the polysaccharide could not explain the increase in xylosyl branching of the glucosyl backbone. From HPAEC-PAD analysis and ESI-MS, the ratio of xylocellotetraosyl:xylocellopentaosyl:xylocellohexaosyl units in the storage XyG is about 2:1:0.2 (Figs. 1B and 2). The storage XyGs are not fucosylated.

Seed storage XyGs isolated from *Tamarindus indica* seed flours yield the expected xylocellotetraosyl oligomeric units upon digestion with *Trichoderma endo*-β-glucanase, whereas those from *H. courbaril* produce an additional group of xylocellopentaosyl and xylocellohexaosyl backbone units (Fig. 2A and B). Electrospray ionization mass spectrometry of these mixtures of XyG oligomers gave fragments *m/z* 1085 (XXXG), *m/z* 1247 (XLXG and XXLG), and *m/z* 1409 (XLLG). The *H. courbaril* storage XyG contain much less of the ion *m/z* 1085, large amounts of ion *m/z* 1247 and a small amount of the ion *m/z* 1409 (Fig. 2B). Although analysis by MS/MS of *m/z* 1247 shows a high proportion of a mixture of XLXG and XXLG, the analysis of the same oligosaccharide mixtures by HPAEC-PAD reveals that XXLG is present in substantial excess in relation to XLXG (Fig. 3). In addition, the *H. courbaril* XyG yields upon digestion substantial amounts of ungalactosylated XXXXG (*m/z* 1379), singly galactosylated XLXXG, XXLXG, or XXXLG (*m/z* 1541), and doubly galactosylated XLLXG, XLXLG or XXLLG oligomers (*m/z* 1703). Although in much lower abundance, fragments compatible with *m/z* ratios of ungalactosylated XXXXXG (*m/z* 1673), singly galactosylated (*m/z* 1835), doubly galactosylated (*m/z* 1997), and even trace amounts of triply galactosylated (*m/z* 2159) xylocellohexameric are present in *H. courbaril* XyG. Although these fragments are also compatible with incompletely digested oligomers, such as XXGXXXG, the chromatographic profiles and known behavior in digestion reactions exclude this possibility. Also, Fanuti, Gidley, and Reid (1991) Tiné, Lima, and Buckeridge (2003) showed that unbranched glucosyl units of the oligosaccharide would be readily attacked by *endo*-β-glucanase, and XXG is not observed.

When *H. courbaril* XyG oligomers are separated by HPAEC, three unidentified oligosaccharides are purified (Fig. 4). These three oligosaccharides represent approximately 30% of the total oligosaccharides produced by *endo*-β-glucanase digestion of XyG. The proportion of monosaccharides, as determined by acid hydrolysis and analysis by HPAEC-PAD, is 5:4:1, (Glc:Xyl:Gal) for all three oligosaccharides, and the linkage analysis shows that all galactosyl residues are non-reducing terminal residues and attached to the O-2 of xylose residues. These data indicate that they differ from each other only by the xylosyl residue to which the galactosyl residue is attached. The linkage analysis of each purified oligomer showed the expected linkages (6-Glc:4,6-Glc:4-Glc) in a ratio 1:3:1, confirming that these oligosaccharides have four xylose residues attached to the O-6 of glucoses for each unbranched

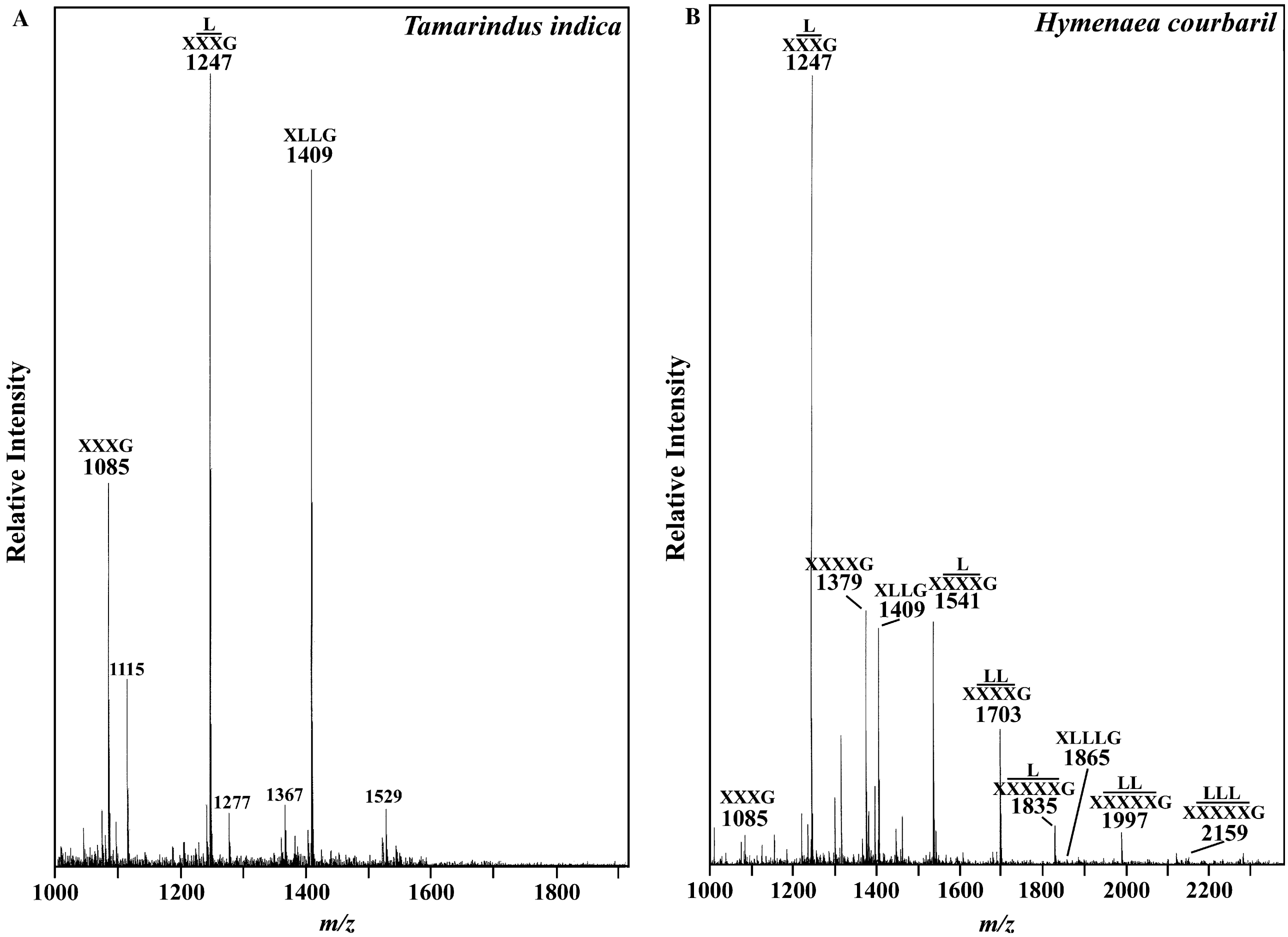

Fig. 2. Electrospray ionization-mass spectrometry of storage XyG oligomers from *Tamarindus indica* (A) and *Hymenaea courbaril* (B). XyG oligomers generated by hydrolysis with *endo*-β-glucanase. The polysaccharide from *T. indica* contains only XXXG-based oligosaccharides, whereas *H. courbaril* contains XXXG-, XXXXG- and XXXXXG-based oligosaccharides in a ratio of 2:1:0.2. If present, the number of galactosyl residues (L) is indicated above the residue, because the precise location cannot be determined by ESI-MS alone.

reducing end. On the basis of these results the expected structure of the three oligosaccharides isolated from a mixture of limit digest oligosaccharides from *Hymenaea* should be XLXXG, XXLXG and XXXLG. However, the position at which galactosyl is linked in each oligosaccharide could not be inferred from their order of elution in the HPAEC-PAD.

Each of the three purified oligosaccharides give *m*/*z* 1541, compatible with the proposed structure of a single galactosylation of an XXXXG oligomer. However, secondary fragmentation of this ion produces different fragments that allowed the identification of the position of the galactosyl residue in each oligosaccharide (Fig. 5). Only the first eluting peak from HPAEC yielded predominantly *m*/*z* 659 (LG) and *m*/*z* 1361 (XXXL) upon secondary fragmentation of *m*/*z* 1541 on either side of the galactosylation site, indicating a galactosyl residue is attached to the fourth Xyl from the non-reducing end (XXXLG). The second eluting peak from HPAEC-PAD yielded the fragment *m*/*z* 773 (XL) and *m*/*z* 1229 (LXXG), which is diagnostic of XLXXG. The third oligosaccharide produced fragments *m*/*z* 1064 (XXL) and *m*/*z* 953 (LXG), consistent with cleavage of the glucan chain on either side of the galactosylated residue in XXLXG.

The identity of XLXXG was confirmed by digestion with a sequence-dependent-galactosidase from cotyledons of *Copaifera langsdorffii* (Alcântara et al., 1999), which hydrolyses galactosyl residues only at the non-reducing end of the oligosaccharide (e.g. XLXG). Upon incubation of the three purified oligomers by this enzyme, only the second eluting peak from HPAEC-PAD could be converted to XXXXG, while the other two remained galactosylated (Fig. 6). The susceptibility of the second oligomer to the enzyme is consistent with the inferred structure from electrospray MS and MS/MS to be XLXXG.

3.2. Distribution of the xylocellotetrameric and xylocellopentameric units in the polymers

Incomplete digestion of the *H. courbaril* XyG with *Trichoderma endo*-β-glucanase gives a complex series of dimers of mostly (galacto)xylocellotetraosyl and (galacto)xylocellopentaosyl backbone units (Fig. 7, Table 1). The dimer T-H is indistinguishable from P-P, but P-H/H-P

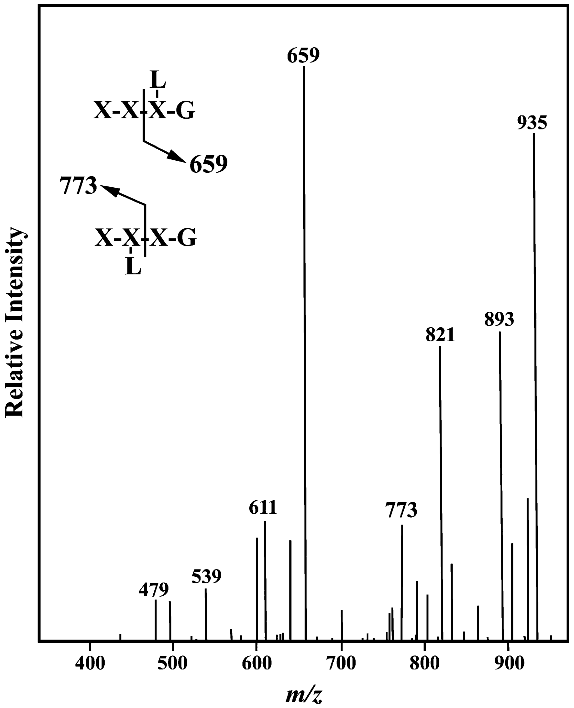

Fig. 3. Fragmentation of the 1247 *m*/*z* trapped after electrospray ionization-mass spectrometry of *Hymenaea courbaril* XyG oligomers generated by hydrolysis with *endo*-β-glucanase. The highly abundant *m*/*z* 659 arises from XXLG, whereas *m*/*z* 773 is from XLXG.

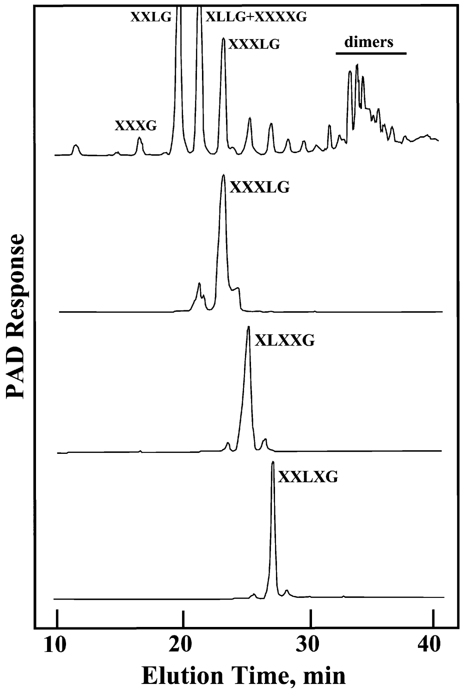

Fig. 4. Analysis by HPAEC-PAD of the oligosaccharides obtained from *Hymenaea courbaril* storage XyGs isolated at full maturity and after hydrolysis with *endo*-β-glucanase. Upper trace, total oligomer profile. Lower traces, analysis of the three single galactosylated XXXXG oligomers after purification.

and H-H dimers are detected in only trace amounts, indicating that P-P units are much more abundant than T-H units. Even though the digestion reaction was carried out in a way to maximize the production of dimers, little bias is observed towards digestion of certain dimers as the ratio of tetrameric and pentameric units is close (1.9:1) to that observed upon complete digestion to monomeric units (2:1). The T-T, T-P/P-T, and P-P dimers were sufficiently abundant to judge intrinsic stoichiometry. The detection of the dimer T-P/P-T (XXXGXXXXG/XXXXGXXXG, *m*/*z* 2423) and a series formed by the addition of one to four galactosyl residues, demonstrated that both series of oligosaccharides are present in the same XyG molecule, indicating they are synthesized by the same synthase-glycosyltransferase complex. The xylocellotetraosyl backbone units are more heavily galactosylated, with over 80% of the T-T dimers bearing at least one galactosyl residue and over 50% bearing two to three galactosyl residues, and those with three galactosyl residues being the most predominant in these dimers. In contrast, the combined T-P and P-T dimers are intermediately galactosylated, and only about one-half of P-P dimers bear more than one Gal residue (Table 1).

The ratio of the T-T:T-P/P-T:P-P dimers was 3.1:2.1:1.0, far from the 4:4:1 (t^2:2tp:p^2) expected for a stochastic assembly of a 2:1 (considering $t = 0.66$ and $p = 0.33$) ratio of T and P monomeric units and an indication that the T and P are not randomly assembled in the polymer. We analyzed a full range of possible directed assemblies of the T and P units to illustrate the basic features required to yield a 3:2:1 ratio (Table 2). We deduced that the 2:1 ratio of T-P/P-T:P-P is supplied most simply by the blocks of T-P-P-T inserted in a polymer of additional T-T units sufficient to give the observed ratio. A model polymer with three P-P units interspersed amongst twelve T units at any spacing satisfies this criterion. However, empirical evidence for synthesis mechanisms is needed because model polymers with the P-P units separated by single T units or simply averaging a P-P unit per four T units, such as a T-P-T + T-P-P-P-T arrangement, also give a 3:2:1 ratio.

This unusual galactosylated mixed-oligomer structure demands a new way of thinking about the biological mechanisms of synthesis of XyGs, their assembly and packing in the wall, and their hydration and mobilization during germination and seedling growth. Ray (1980) and Hayashi and Matsuda (1981) first demonstrated the coordinate addition of Glc and Xyl from their respective nucleotide-sugars and Golgi membranes to generate polymers containing isoprimeverose and higher order oligomers. They and others (Farkas & Maclachlan, 1988; Gordon & Maclachlan, 1989) optimized synthesis to show that significant amounts of XXXG were made at high substrate concentrations and proposed that cellobiose was the basic unit of synthesis, primarily because of the repeating cellotetrameric unit. The cellobiose unit assembly is attractive because it overcomes the steric problem imposed by a (1 → 4)-β-linkage due to the inversion of each glycosyl residue nearly

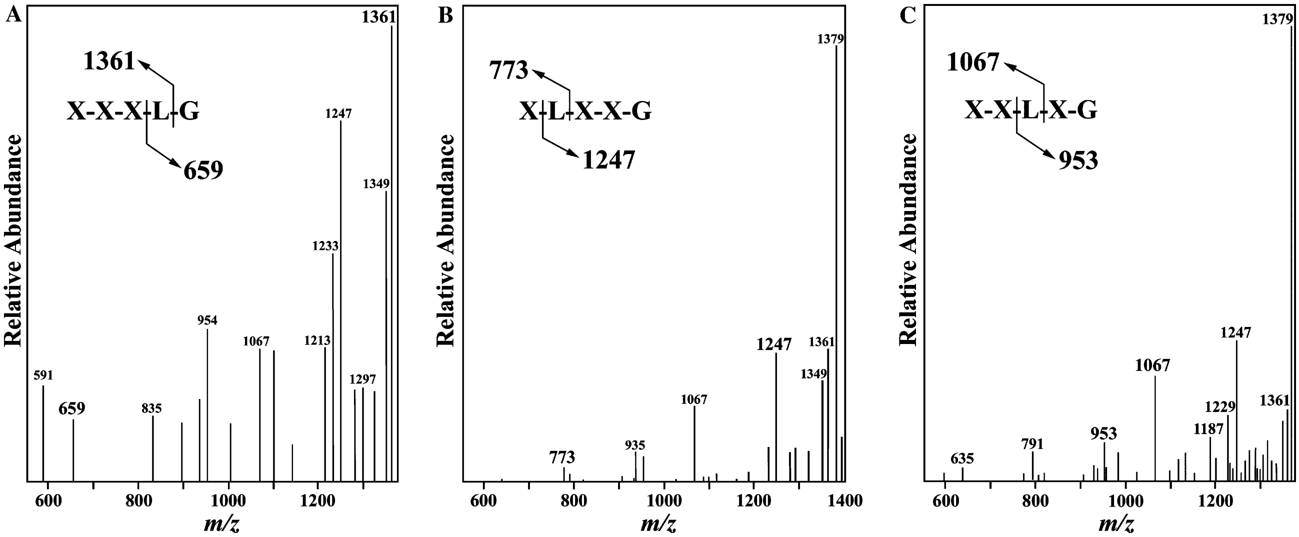

Fig. 5. Electrospray ionization-mass spectrometry of the purified oligosaccharides from *H. courbaril* storage XyG. The locations of the single galactosyl residues in each oligosaccharide are identified by the cleavage patterns revealed by MS/MS of *m*/*z* 1541. (A) Oligomer XXXLG yields diagnostic *m*/*z* 659 and *m*/*z* 1361. (B) Oligomer XLXXG yields diagnostic oligomers *m*/*z* 773 and *m*/*z* 1247. (C) Oligomer XXLXG is confirmed by the presence of diagnostic oligomers *m*/*z* 953 and *m*/*z* 1067.

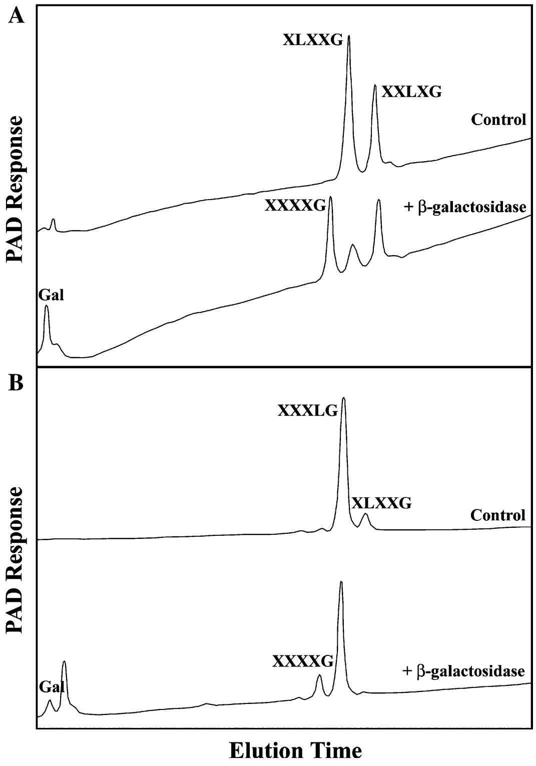

Fig. 6. Digestion of the *H. courbaril* singly galactosylated XXXXG oligosaccharides with *C. langsdorffii* β-galactosidase. Biogel P-6 fractions. Only oligosaccharide XLXXG generated XXXXG (A) and free galactose (Gal).

180° with respect to its neighbor (Carpita & Vergara, 1998; Carpita et al., 1996). While the appearance of xylocellopentaosyl in the storage XyG appears to refute this hypothesis, the higher order arrangement of T-P-P-T not only preserves the cellobiosyl unit mode of polymerization but also indicates that the synthase complex may be much larger than expected.

Faik et al. (1997) found a pea galactosyl transferase with a preference to add to the xylosyl residue closest the reducing end of the oligomer, transforming XXXG into XXLG. Evidence to date supports such specificity whereby each residue subtending the glucan chain requires a separate enzyme for each xylosyl residue (Faik, Price, Raikhel, & Keegstra, 2002) and galactosyl residue (Madson et al., 2003). Such specificity suggests that the *Hymenaea* storage XyG complex retains up to 18 glucosyl residues that can be read for addition of xylosyl and galactosyl units at precise positions. Close inspection of XyG oligomeric profile of XyGs from tamarind (Fig. 2) and Arabidopsis (not shown) by ESI-MS gave no trace of these higher order oligomers, indicating that the XyG synthase complex of *H. courbaril* uniquely recruited additional xylosyl transferases to produce the pentameric and hexameric backbone units.

As originally suggested by Tiné, Cortelazzo, and Buckeridge (2000), the strict xylocellotetraose unit assembly of the primary cotyledon walls compared to the mixed tetrameric–pentameric assembly of the storage polymer indicate that the cotyledons of *H. courbaril* have at least two kinds of XyG synthase complexes. Distinct fucosylated XyG domains in the growing wall and the unfucosylated storage XyG domains in those walls further suggests expression of two distinct synthase complexes during development (data not shown). During seed maturation, the shift from the structural to the storage XyG in the parenchyma cells possibly involves changes in the biosynthetic complex, probably with a shift in the expression of different glucosyl-, xylosyl-, and galactosyl-transferase genes (Perrin, Wilkerson, & Keegstra, 2001).

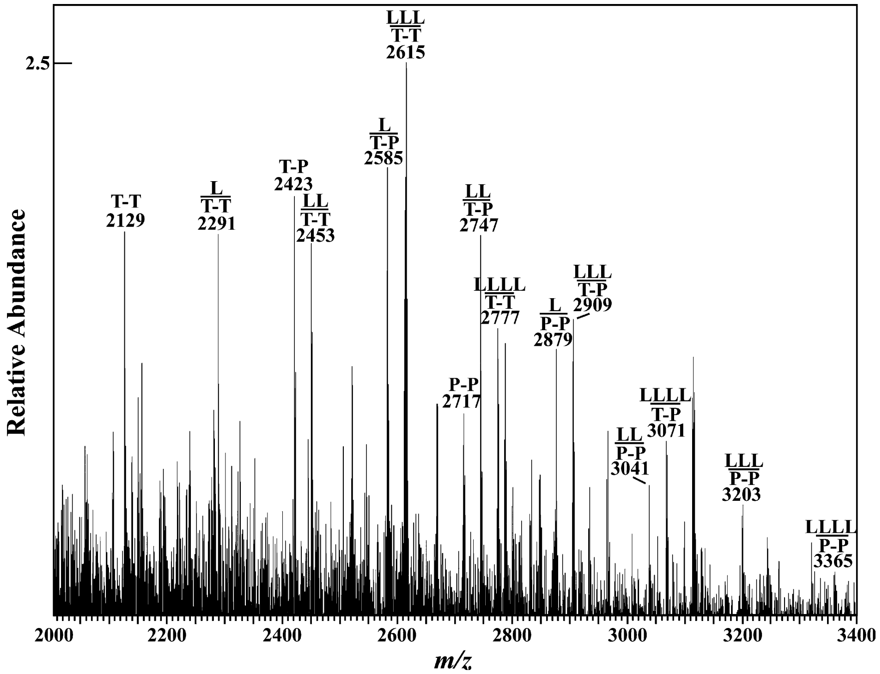

Fig. 7. Electrospray ionization-mass spectrometry of dimer units of (galacto)xylocellotetraosyl (T) and (galacto)xylocellopentaosyl (P) oligomers from *Hymenaea courbaril*. XyG oligomers generated by limited hydrolysis with *endo*-β-glucanase. If present, the number of galactosyl residues (L) is indicated above each dimer pair, because the precise location cannot be determined by ESI-MS alone.

Table 1
Distribution of dimers of *Hymenaea* XyGs and the relative proportions of galactosylated residues based on electrospray MS

	XXXG-XXXG	XXXG-XXXXG	XXXXG-XXXXG
Molar ratio	3.06	2.14	1.00
Proportion of Gal residues (Mole%)			
0	0.17	0.27	0.34
1	0.16	0.21	0.29
2	0.19	0.20	0.13
3	0.35	0.21	0.18
4	0.13	0.11	0.06
5	–	n.d.	n.d.
6	–	–	n.d.

n.d., not detected.

Table 2
Stochastic and directed arrangements of xylocellotetraosyl and xylocellopentaosyl units in xyloglucan polymers

Model polymer	T-T:T-P + P-T:P-P
T-T-**P**-T-T-T-**P**-**P**-T-T-T-**P**-**P**-T-T-**P**-T-T	8:8:2
T-**P**-T-T-**P**-T-T-**P**-T-T-**P**-T-T-**P**-T-T-**P**-T	6:12:0
T-T-T-T-T-T-**P**-**P**-**P**-**P**-**P**-**P**-T-T-T-T-T-T	11:2:5
T-T-T-**P**-**P**-**P**-T-T-T-T-T-T-**P**-**P**-**P**-T-T-T	10:4:4
T-T-**P**-**P**-T-T-T-T-**P**-**P**-T-T-T-T-**P**-**P**-T-T	9:6:3
T-T-**P**-**P**-T-T-**P**-**P**-T-T-**P**-**P**-T-T-T-T-T-T	9:6:3
T-T-**P**-**P**-T-**P**-**P**-T-**P**-**P**-T-T-T-T-T-T-T-T	9:6:3
T-T-**P**-T-T-T-**P**-**P**-T-T-T-**P**-**P**-**P**-T-T-T-T	9:6:3

Stochastic (random) arrangements give a 4:4:1 ratio of dimers based on a xylocellotetraose:xylocellopentaosyl ratio of 2:1. The 3:2:1 ratio observed demands a more directed arrangement. Arrangements containing the repeating unit T-P-P-T spaced by additional T units gives such a ratio; when considering that the *endo*-β-glucan hydrolase may cleave between each unit oligomer, the tetramer T-P-P-T will always give a 2:1 ratio of T-P + P-T:P-P. The three P-P units placed anywhere within the stretch of twelve T units gives the 3:2:1 ratio. Ps are in bold to show clearly their position in the sequences and distinguish them from Ts.

3.3. Implications of fine structure for xyloglucan properties

A XyG molecule with a mixture of blocks of T and P units in the same molecule alters the three-dimensional structure of XyGs, primarily with respect to the positioning of the galactosyl residues along the main chain. The fifth glucose inverts the chain, inverting the position of the galactose in the next oligosaccharide when compared to the previous oligosaccharide. This leads to a uniform distribution of galactoses, even in a polymer not so rich in XLLG and rich in XXLG, like the one in *H. courbaril.* As the P-P units are relatively less galactosylated than the T-T units, these particular dimers may constitute regions with different local properties that could affect solubility, packing during assembly and, ultimately, hydrolysis of the polymer after hydration during seed germination.

H. courbaril XyGs pack more tightly with cellulose (Freitas et al., 2005; Lima & Buckeridge, 2001; Lima, Loh, & Buckeridge, 2004), are hydrolyzed more quickly by *endo*-β-glucanase than the xylocellotetraosyl-based tamarind/copaifera XyGs (Tiné et al., 2003), and produce less stable aqueous solutions (M. Minhoto and M.S. Buckeridge, unpublished observations). One possible explanation for these different properties is that combinations of the T and P units increase the distance between subtending galactoses in regions of the polymer in a way that does not compromise shape and solubility. Such a structure would have longer domains that could be more interactive with other XyG molecules or cellulose and explain, at least in part, the higher interaction with cellulose observed for *Hymenaea* XyG (Lima & Buckeridge, 2001; Lima et al., 2004). The XL motifs are associated with higher cellulose-binding capacity (Lima & Buckeridge, 2001). However, we have observed that the manipulation of solutions of *Hymenaea* XyG in the laboratory requires more attention, because it is not as stable as the solutions of the other galacto-xylocellotetraosyl-based storage XyGs. After a few days, *Hymenaea* XyG usually precipitates from solution forming insoluble aggregates. Indeed, it has been demonstrated that loss of galactosyl residues from XyG also changes the physical state and lowers solubility (Shirakawa, Yamatoya, & Nishinari, 1998; Yamanaka et al., 1999).

3.4. Implications of fine structure for xyloglucan disassembly

The reserve function of xyloglucan in cotyledons has been demonstrated for seeds of *T. majus*, *T.indica*, *C. langsdorffii*, *H. courbaril* (Buckeridge et al., 2000). Reid and co-workers (for a review, see Buckeridge et al., 2000) isolated the four main enzymes responsible for xyloglucan degradation in *T. majus*. The *H. courbaril* storage XyG is hydrolyzed during seedling development by the action of XET, β-galactosidase, α-xylosidase and β-glucosidase (Tiné et al., 2000). The mobilization pattern in *H. courbaril* is comparable to other known seed storage mobilization systems, such as the one of *Tropaeolum majus* (Edwards, Dea, Bulpin, & Reid, 1985), *T. indica* (Reis, Vian, Darzens, & Roland, 1987) and *C. langsdorffii* (Buckeridge et al., 1992). The activity of the *C. langsdorffii* β-galactosidase on XLXXG confirmed that the enzyme has high specificity to "XL" motifs positioned at the non-reducing end of both T and P units. As the disassembly via α-xylosidases and β-glucosidases of oligomers proceeds from the non-reducing end, substitutions at the reducing end or elsewhere along the length of the oligosaccharide chain seem to have little or no effect on the recognition of the substrate by relevant hydrolases (Tiné et al., 2000).

The proportion of oligomers with β-galactosidase-sensitive XL structures is approximately 24% in *H. courbaril*, whereas the amount of galactose released by the β-galactosidase from the total mixture of oligosaccharides is negligible (data not shown). This indicates that XyG disassembling mechanism in *H. courbaril* is slightly different from that of other XLLG-rich seeds, such as copaifera, tamarind and nasturtium. Once the oligosaccharide is released by XyG *endo*-transglucosylase (XET) activity in the apoplast, the first enzyme that acts upon it is the α-xylosidase. The unusual branching pattern could also lead to different kinetics of interaction of an XET with the polysaccharide (Tiné et al., 2003; Vincken et al., 1997). As Levy, Maclachlan, and Staehelin (1997) described, the backbone can exist in twisted and straightened conformations, and XLXG and XXFG are particularly effective in maintenance of the backbone in the straightened conformation, suggesting that it facilitated binding to cellulose. However, Peña, Ryden, Madson, Smith, and Carpita (2004), using Arabidopsis mutants with grossly altered XyG structures, found that XyGs enriched with XLXG and XXFG were the best substrates for XET activity. Thus, if the computer simulations of Levy et al. (1997) are correct, then the chain straightening function may enhance the binding of any member of the XTH family to XyG. The behavior of polymeric substrates is, to a certain extent, consistent with the activity of oligomeric substrates, where XLLG is a significantly better acceptor than either XXXG or XXFG (Purugganan, Braam, & Fry, 1997), indicating a strong preference toward substrate and acceptor with Gal residues on the penultimate Xyl. Therefore, in *H. courbaril*, where XL motifs are less common due to the predominance of XXLG and XXXXG the storage XyG is probably a better substrate for XET. This might also explain why the XET from *H. courbaril* is so different from that of nasturtium (Tiné et al., 2000).

3.5. Functional implications for a novel type of xyloglucan

Structurally, the storage XyGs of *H. courbaril* differ from copaifera, nasturtium and tamarind storage XyGs in at least two ways: they possess an oligomer profile with one more isoprimeverose unit and the amounts of β-galactosidase-sensitive XL- termini upon XET or XEH action is lower. The impact of these structural features on the physicochemical properties of the storage XyG still needs to be investigated, but the presence of a fifth glucose in the main chain inverts the position of the side chains and creates a unique pattern of xylosyl and galactosyl side chain, without changes in the proportion of the monosaccharides (Buckeridge et al., 1997). It may be speculated that XyG has two principal functional molecular domains: XL domains associated with intermolecular binding and LG domains that are associated with hydrolysis. This finding, together with the high molecular weight observed for storage XyGs (Franco, Rodrigues, Serra, Panegassi, & Buckeridge, 1996), which is likely to be related to a large agglomerate of lower molecular weight molecules (Freitas et al., 2005), suggests that the fine structural features of the polymer from *H. courbaril*, with its much less uniform distribution of galactoses around the main glucose chain, might have evolved towards a production of a maximum chain–chain interaction, but still preserving a galactose positioning that affords water solubility. These two properties would guarantee the formation of a storage wall with very little cellulose and possibly with a higher packing level in comparison with the XXXG-only based polymers.

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