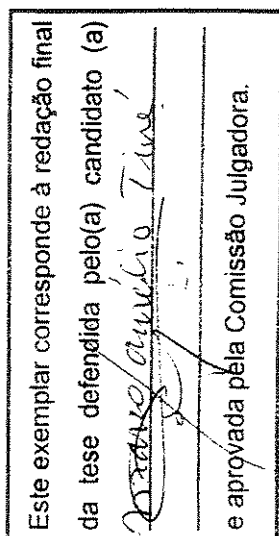


UNIVERSIDADE ESTADUAL DE CAMPINAS



Marco Aurélio Silva Tiné

O conteúdo informacional da molécula de xiloglucano de cotilédones de *Hymenaea courbaril* reflete suas funções em nível celular.



Tese apresentada ao Instituto de Biologia para obtenção do título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

Orientador Dr. Marcos Silveira Buckeridge

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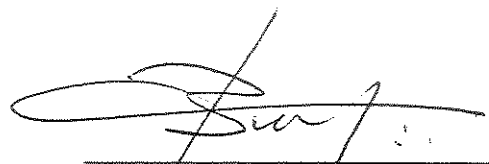
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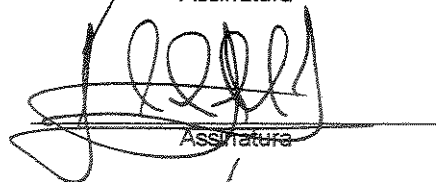
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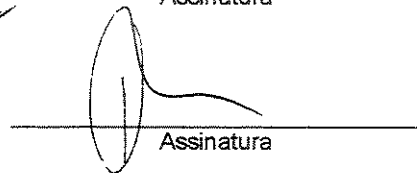
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Resumo

Sementes de algumas espécies apresentam espessamentos de parede celular devido à deposição de um determinado polissacarídeo que* constitui uma reserva de carbono e energia para a plântula após a germinação. Um destes polissacarídeos é o xiloglucano (XG). Este polissacarídeo apresenta uma cadeia principal de glucose β -(1,4) ligada com ramificações regulares com xilose α -(1,6) ligada. Algumas das xiloses apresentam ainda ramificações com galactose β -(1,2) ligada. Na parede primária algumas das galactoses possuem ainda ramificações de fucose α -(1,2) ligadas. Dentre as sementes que possuem esta reserva estão *Tamarindus indica*, *Tropaeolum majus*, *Copaifera langsdorffii* e *Hymenaea courbaril*.

Uma dos principais métodos de análise de xiloglucano é a digestão do polissacarídeo com celulase e a análise dos oligossacarídeos resultantes. Nas espécies estudadas os oligossacarídeos liberados possuem quatro glucoses na cadeia principal, mas em *H. courbaril*, foi descrita uma série de oligossacarídeos com cinco glucoses na cadeia principal, além da série habitual de quatro glucoses. Três novos oligossacarídeos constituintes do xiloglucano de reserva de *H. courbaril* foram isolados e caracterizados neste trabalho. Os oligossacarídeos possuem apenas uma galactose cada e diferem entre si pela posição desta galactose. Como a presença de oligossacarídeos com cinco glucoses só foi descrita até hoje nesta espécie, a interação entre a xilosil e a glucosil-transferase no Complexo de Golgi durante a biossíntese da parede de reserva de *H. courbaril* pode vir a ser uma importante ferramenta para entender o processo de biossíntese do polissacarídeo. A presença desta nova série de oligossacarídeos provavelmente inverte a posição das ramificações de xiloglucano. Esta inversão da cadeia poderia alterar a simetria da molécula, o que poderia ter implicações na interação com outros polissacarídeos e enzimas do seu próprio metabolismo.

Um estudo da interação da celulase com este polissacarídeo mostrou que a velocidade de degradação do xiloglucano de *H. courbaril* é maior que a degradação do xiloglucano de *C. langsdorffii*. O padrão de ramificação do polímero com galactose

também interfere no padrão de ataque da celulase sobre o polímero, sendo que a redução das ramificações aumenta a suscetibilidade do polissacarídeo à enzima. A presença de uma exo-hidrolase (β -galactosidase) durante a hidrólise pela celulase altera o estado final de equilíbrio mesmo não participando diretamente na reação da celulase, o que indica uma complexa interação entre as enzimas do metabolismo de xiloglucano que poderia ocorrer através do próprio polímero na forma de alterações nas cadeias laterais. Os processos de degradação e biossíntese do xiloglucano, portanto, parecem estar finamente sincronizados e o veículo para a integração entre os dois processos poderia ser o próprio polissacarídeo, uma vez que o padrão de ramificação do xiloglucano é capaz de codificar informação suficiente para modular a ação das enzimas sobre o xiloglucano.

A localização sub-celular do resíduo de fucose na parede de reserva mostrou que o polissacarídeo de reserva é depositado em uma camada entre duas camadas estruturais. A parede de reserva, portanto, possui pelo menos duas populações distintas de xiloglucano que diferem entre si pela posição, estrutura e função: o xiloglucano de reserva é mais solúvel, pobre em fucose e depositado em uma camada intermediária da parede; o xiloglucano estrutural é menos solúvel, fucosilado e depositado em volta da camada de reserva. A presença da camada estrutural externa deve ser um importante elemento estrutural do tecido durante a mobilização do xiloglucano de reserva e a diferença na composição dos polissacarídeos permite que o xiloglucano de reserva seja desmontado pelas exo-hidrolases sem afetar o xiloglucano estrutural. As diferenças na composição das duas populações de xiloglucano, portanto, refletem diferenças fisiológicas fundamentais e o padrão de ramificação parece ser um elemento importante para manter as diferenças entre os xiloglucanos de forma a permitir que a parede funcione como reserva de polissacarídeos.

Abstract

Seeds of some species have cell wall thickenings of a single polysaccharide that is considered as a reserve of carbon and energy to the plantlet after germination. One of these polysaccharides is xyloglucan (XG). This polymer has a main chain of glucose β -(1,4) linked with regular branches of α -(1,6) xylose. Some of the xyloses have side chains of β -(1,2)-linked galactoses. In the primary cell wall, some of these galactoses are further branched with α -(1,2)-linked fucose. Among the seeds that accumulate this reserve are: *Tamarindus indica*, *Tropaeolum majus*, *Copaifera langsdorffii* e *Hymenaea courbaril*.

One of the main methods of analysis of xyloglucan is the digestion with fungal cellulase and analysis of the oligosaccharide produced. In all the species studied so far, the oligosaccharides produced have four glucoses in the main chain but in *H. courbaril*, a series of oligosaccharides with five glucoses in the main chain was reported besides the usual series with four glucoses. Three new oligosaccharides from the storage xyloglucan of *H. courbaril* were isolated and characterised in this work. Since the presence of this new series of oligosaccharides have only been described in this species, the interaction between the xylosyl and the glucosyl-transferase in the Golgi Complex during the biosynthesis of the storage wall of *H. courbaril* may become an important tool to understand the biosynthesis process. The presence of this new series probably inverts the position of the xyloglucan side-chains and this inversion might alter the symmetry of the molecule and this would have implications in the interaction with other polysaccharides and the enzymes.

The study of the interaction of *Trichoderma* cellulase with this polymer showed that the speed of the degradation of the xyloglucan of *H. courbaril* is higher than the degradation of the xyloglucan from *C. langsdorffii*. The branching pattern with galactose also interferes in the attachment of the cellulase to the polymer: the removal of the side chains also modulates the interaction between the enzyme and the substrate. The presence of an exo-hydrolase (β -galactosidase) during the hydrolysis by cellulase alters the final equilibrium reached by the reaction even without direct participation in the

cellulase reaction, what suggests a complex interaction between the enzymes of xyloglucan metabolism that may occur through the polysaccharide itself as changes in its side-chains. The biosynthetic and disassembling processes, therefore, seem to be fine tuned and the form to the integration of these two processes could be the xyloglucan, since its branching pattern is capable to code enough information to modulate the action of the enzymes upon the polysaccharide.

The sub-celular localisation of the fucosyl residue in the storage wall showed that the storage polysaccharide is deposited in a layer between two structural layers. The storage wall, therefore, has at least two distinct populations of xyloglucan that differ from each other in position, structure and function: the storage xyloglucan is more soluble, poor in fucose and deposited in an intermediate layer in the wall; the structural xyloglucan is less soluble, fucosylated and deposited around the storage layer. The presence of an outer structural layer is probably an important element in the structure of the tissue during the mobilisation of the storage xyloglucan and the difference in composition between the polymers allows the disassembling by the exo-xydrolases with little or no effect on the structural xyloglucan. The differences in the composition of the two populations of xyloglucan, therefore, reflect fundamental physiological differences and the branching pattern seems to be an important element for maintenance of storage xyloglucan function.

Introdução

Parede celular

Funções e composição

Todas as células vegetais possuem uma parede celular. Esta estrutura é a principal responsável pela capacidade de resistir a esforços mecânicos e, portanto, pelas propriedades mecânicas dos tecidos. Por restringir a expansão do citoplasma, a parede celular também determina a forma da célula e sua taxa de crescimento (Carpita e Gibeaut, 1993). A parede celular, portanto, é um dos elementos-chave no crescimento e diferenciação de toda a planta.

Por possuir polímeros ácidos, a parede de células vegetais em crescimento (parede primária) participa ainda na manutenção do ambiente iônico da célula modulando não apenas a passagem de íons, mas também de espécies químicas neutras. A parede participa ainda dos processos de defesa contra a invasão de patógenos, sinalização e controle do potencial hídrico da célula (Street e Öpik, 1984, Waldron e Brett, 1990). Cada uma destas funções é exercida em graus diferentes de acordo com o tecido do qual a célula faz parte.

Essa diversidade de funções se reflete na diversidade de composição da parede. Segundo o modelo corrente (Carpita e Gibeaut, 1993), a parede primária é composta por 3 domínios independentes que possuem composição e funções distintas: pectina, celulose-hemicelulose e proteínas:

- As pectinas são polissacarídeos ricos em ácido galacturônico que modulam a permeabilidade da parede a solutos (iônicos ou não), aderem as células umas às outras e seus fragmentos estão entre as moléculas mais eficientes na sinalização de infecção por patógenos (Dietrich et al., 1998). Vários polímeros podem participar da composição deste domínio (homogalacturonano, ramnogalacturonanos I e II, galactanos, arabinogalactano), mas a composição exata varia de tecido para tecido.

- A matriz celulose-hemicelulose é a principal responsável pela resistência mecânica da parede e, portanto, de todo o tecido. As microfibrilas de celulose são depositadas na parede sendo orientadas pelo citoesqueleto e definem a direção em que a parede irá se estender (a extensão da parede ocorrerá principalmente no sentido perpendicular ao da deposição das microfibrilas). A ligação entre as microfibrilas é feita por hemiceluloses que envolvem as microfibrilas. De modo geral, a deposição de celulose define a direção do crescimento, enquanto o metabolismo das hemiceluloses define a taxa de crescimento. Embora a celulose seja a mesma em todos os vegetais, a composição das hemiceluloses pode variar e, assim como nas pectinas, vários polissacarídeos são descritos como hemiceluloses: (galacto)mananos, xiloglucanos (XG), glucanos de ligação mista, (arabino)xilanos. Nem todas as células possuem todos estes polímeros e a composição exata da parede pode variar de célula para célula e até mesmo de uma parte da célula para outra.

- A matriz protéica pode ser subdividida em proteínas estruturais e enzimas. Várias proteínas estruturais já foram descritas como, por exemplo, as proteínas ricas em hidroxiprolina e as extensinas, mas suas funções ainda não são claras. As enzimas participam do metabolismo dos polissacarídeos de parede já referidos e, portanto, são importantes elementos em todos os processos citados acima. Diversas hidrolases e transferases podem ser extraídas da parede, mas quais estão presentes em um determinado tecido depende de quais polissacarídeos compõem a parede da célula. Isso confere à parede um rico espectro de possibilidades metabólicas, adequado à diversidade de funções e condições metabólicas às quais esta organela pode ser exposta.

Embora amplamente aceito, este modelo não considera a presença de compostos fenólicos na parede. Sua ausência no modelo ignora a possibilidade de formação de ligações cruzadas entre os polissacarídeos (McNeil et al., 1984), além de possíveis ligações de polissacarídeos às proteínas (McCann e Roberts, 1991, Liyama et al., 1994). Carpita e Gibeaut (1993) supõem que estes domínios sejam essencialmente independentes, ao contrário do que sugerem outros autores (Coimbra et al., 1994 e Goonerate et al., 1994).

Além destas funções típicas de parede primária, a parede celular vegetal pode apresentar especializações. As paredes podem enrijecer e lignificar em tecidos de transporte e sustentação como o xilema, acumular suberina ou camadas adicionais da matriz celulose-hemicelulose como em células guarda de estômato (Carpita e McCann, 2000). Em sementes, pode ocorrer o acúmulo de alguns polissacarídeos específicos que constituem uma reserva de carbono para o crescimento da plântula após a germinação (Bewley e Black, 1978; Buckeridge e Reid, 1996).

Entre os polissacarídeos que podem ser utilizados como reserva estão o manano, o galactomanano, o galactano e o xiloglucano. Exceto pelo galactano, que constitui uma ramificação de cadeias de pectina, os demais polissacarídeos são hemiceluloses. Esta especialização provavelmente surgiu através de uma separação temporal dos processos de catabolismo e anabolismo dos polissacarídeos da parede que ocorrem normalmente nas paredes primárias (Buckeridge et al., 2000a). Nas células em crescimento os polissacarídeos estão em constante modificação para assegurar o crescimento controlado da célula. À medida que os polímeros antigos são modificados para aliviar as tensões oriundas da distensão da parede, novas moléculas são incorporadas à parede garantindo a integridade da célula. Nos tecidos de reserva, um dos polissacarídeos de parede é sintetizado em grande quantidade durante a maturação da semente sem que haja aumento da sua taxa de degradação. Com isso, a semente quiescente acumula um grande depósito de polissacarídeos nas paredes das células parenquimáticas. Após a germinação, ocorre um grande aumento no catabolismo deste polímero na parede e os monossacarídeos constituintes são absorvidos pela célula e utilizados como fonte de carbono para o crescimento.

Por apresentarem grande teor destes polissacarídeos, as sementes são utilizadas na indústria como fonte desses polímeros. Na indústria alimentícia, por exemplo, estes polissacarídeos são amplamente utilizados como espessantes de baixo valor calórico (gomas) e sua ingestão aumenta o consumo de fibras alimentares, trazendo vários benefícios a quem as consome (Buckeridge e Tiné, 2001). Os polissacarídeos mais amplamente utilizados são o xiloglucano e o galactomanano, acumulados em sementes de algumas espécies de leguminosas.

Xiloglucano: estrutura, função e metabolismo na parede primária

Xiloglucano é uma das principais hemiceluloses de dicotiledôneas. Sua cadeia principal é composta de glucose¹ ligada $\beta(1,4)$. Esta cadeia possui ramificações regulares com xilose $\alpha(1,6)$ ligadas e algumas destas xiloses possuem ainda ramificações com galactose ligada $\beta(1,2)$. Em parede primária, pode haver ainda uma fucose ligada à galactose através de ligações $\alpha(1,2)$, mas esta substituição não ocorre no xiloglucano de reserva. Pela nomenclatura corrente (Fry et al., 1993), os resíduos de glucose não ramificados são denominados **G**, enquanto os pontos da cadeia principal ramificados por xilose são denominados **X** e quando há ramificação com galactose, o trissacarídeo glucose-xilose-galactose é denominado **L** (Figura 1). Na parede primária pode ocorrer ainda ramificação de fucose $\alpha(1,2)$ ligada na galactose e neste caso o tetrassacarídeo é denominado **F**. Outras modificações podem ocorrer ainda na parede primária como arabinose (Keifer et al., 1990), duas xiloses em uma mesma glucose (Hisamatsu et al., 1992), ácido ferúlico (Ishii et al., 1990) e acetilação nas galactoses (Kato e Matsuda, 1985; Hayashi, 1989).

Nas paredes primárias o xiloglucano está intimamente ligado à celulose, chegando inclusive a penetrar na microfibrila (Baba et al., 1994). O comprimento da cadeia de xiloglucano possibilita que uma mesma molécula estabeleça ligações com mais de uma microfibrila de celulose, formando ligações cruzadas entre as mesmas, o que aumenta ainda mais a resistência da parede às forças externas (Hayashi e MacLachlan, 1984). Além de estabelecer ligações entre as microfibrilas, o revestimento

¹ Neste trabalho, a nomenclatura dos carboidratos (principalmente polissacarídeos) será utilizada de forma a facilitar a compreensão e agilizar a distinção entre compostos similares. Ao invés de glicose, será utilizado o termo glucose. Isto visa distinguir termos como glicosídeos (um aglicon ligado a um açúcar qualquer) de glucosídeos (um aglicon, ligado a uma molécula de glucose). Esta designação permite utilizar o termo geral glicano(s) para polissacarídeos compostos de qualquer mistura de monossacarídeos, ao contrário do termo glucanos, que será usado para designar polissacarídeos compostos inteira ou predominantemente por moléculas de glucose, tais como a celulose, amido, glucanos de ligação mista ou xiloglucanos.

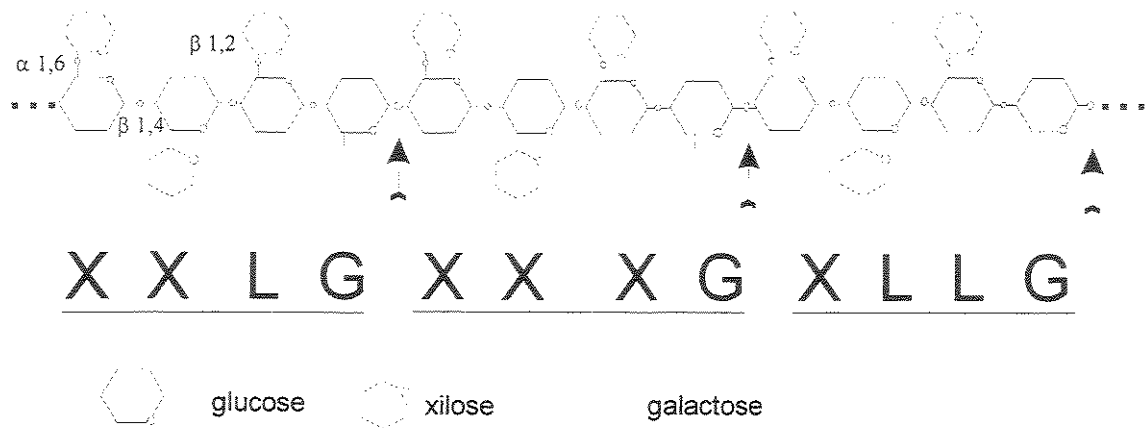


Figura 1. Representação esquemática de uma molécula de xiloglucano de reserva. Os tipos de ligações estão indicados no lado esquerdo da molécula. As setas representam os sítios de ataque da celulase. Os fragmentos gerados pela digestão deste segmento hipotético de xiloglucano com celulase de *Trichoderma viride* seriam: XXL, G, XXX, G e XLL, G, segundo a nomenclatura corrente (Fry et al., 1993). Esquema adaptado de Driouich et al. (1994).

de xiloglucano mantém a organização das microfibrilas impedindo que estas coalesçam e formem feixes muito espessos (McCann et al., 1990).

Como a maioria dos polissacarídeos de parede celular, o xiloglucano é sintetizado no complexo de Golgi. A cadeia principal de glucose e as ramificações de xilose são sintetizadas simultaneamente na face cis da organela, enquanto as galactoses são adicionadas na face trans da organela (ver Driouich et al., 1994 para uma revisão). Na parede primária, a fucose é adicionada na rede trans do Complexo de Golgi pouco antes de sua exportação para a parede celular (Figura 2). O polissacarídeo é montado por transglicosilação utilizando nucleotídeos-açúcares (principalmente UDP-açúcares) como doadores (Gibeaut, 2000). As vesículas provenientes do complexo de Golgi que contêm o xiloglucano se fundem com a membrana plasmática enriquecendo o apoplasto com estas moléculas. Algumas destas moléculas se infiltram na fibrila de celulose enquanto esta está sendo sintetizada criando regiões amorfas na microfibrila. Em seguida a microfibrila recém sintetizada permeada de xiloglucano é integrada à parede com a

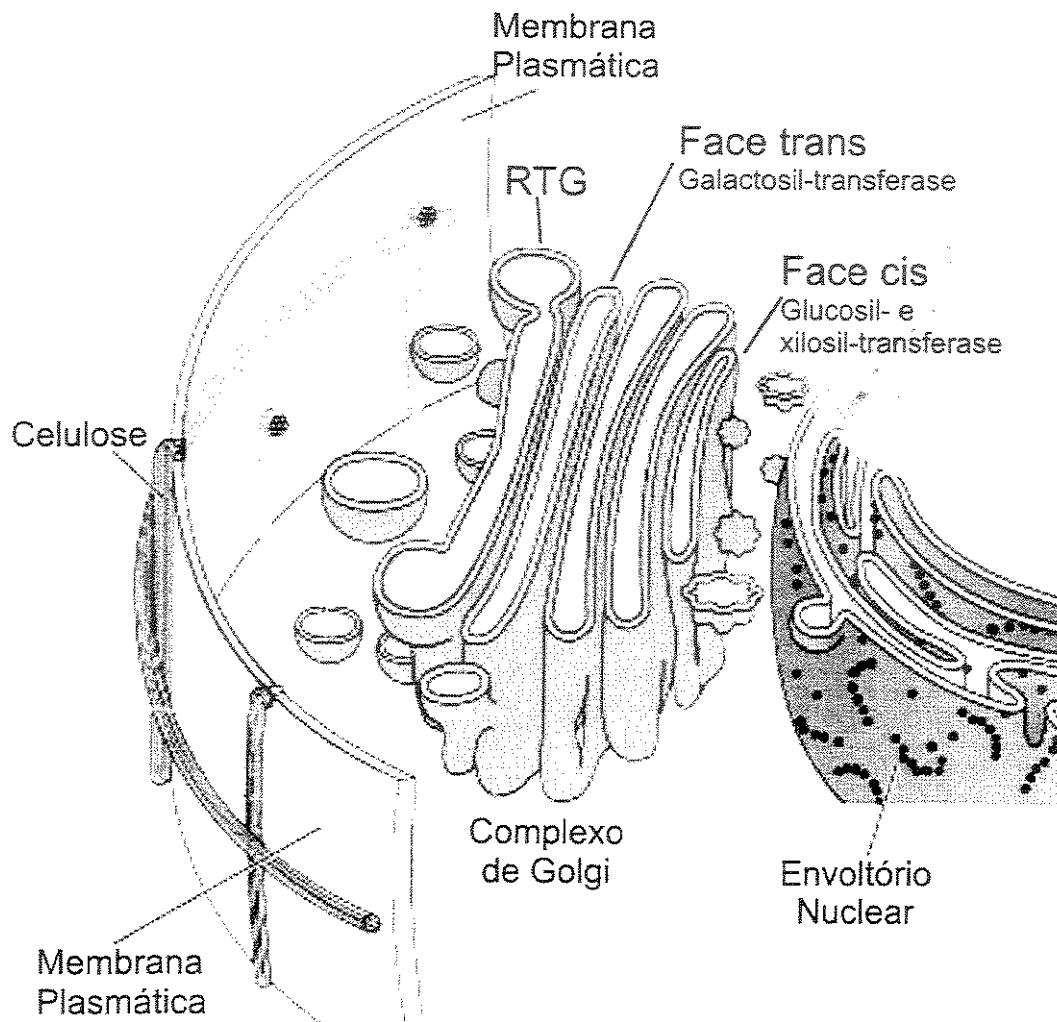


Figura 2. Localização proposta para as glicosil-transferases responsáveis pela biossíntese de xiloglucano no complexo de Golgi. Na face cis é montado o esqueleto principal de glucose e xilose. A galactose é adicionada na face trans e a fucose (no caso do xiloglucano de parede primária) é adicionada na rede trans do Golgi (RTG). Adaptado de Carpita e McCann (2000).

formação de ligações entre os fragmentos de xiloglucano de microfibrilas adjacentes (Hayashi, 1989). A dificuldade de solubilizar enzimas trans-membrânicas sem perda de atividade dificulta o isolamento e caracterização das glicosil-transferases envolvidas na biossíntese do xiloglucano. O primeiro gene de biossíntese de polissacarídeo de parede (celulose) foi identificado por homologia com o gene de síntese de celulose de bactérias e clonado apenas em 1996 (Pear et al., 1996). Acredita-se que haja uma glicosil-

transferase para cada ligação adicionada aos polímeros sintetizados no Complexo de Golgi. Neste caso, pelo menos três glicosil-transferases estariam envolvidas na síntese de xiloglucano de reserva: glucosil-transferase, xilosil-transferase e galactosil transferase. Até o momento, a única glicosil-transferase relacionada à síntese de xiloglucano clonada é a fucosil-transferase (Perrin et al., 1999) mas esta enzima não deve estar ligada à síntese de xiloglucano de reserva, uma vez que o polissacarídeo de reserva não é fucosilado. Apesar desta dificuldade em isolar as proteínas, estudos de cinética enzimática têm sido realizados com preparações de membrana de complexo de Golgi (Faïk et al., 1997, Perrin et al., 1999). Os dados iniciais de K_m das enzimas obtidos nestes trabalhos sugerem que o controle da taxa de síntese de polissacarídeo poderia ser feito (ao menos em parte) pelo controle da concentração de UDP-glucose e UDP-xilose na face cis do Golgi, uma vez que o K_m destas transferases é maior que o da galactosil-transferase. No caso dos glucanos de cadeia mista (β -(1,3), β -(1,4)) de milho, por exemplo, a estrutura do polissacarídeo também parece ser regulada pela disponibilidade de substrato para a glucosil-transferase (Buckeridge et al., 1999).

Uma das formas mais usuais de estudo do xiloglucano é a hidrólise do polímero com uma endo- β -glucanase e análise dos oligossacarídeos resultantes. O padrão de oligossacarídeos gerados por digestão com celulase varia de acordo com a espécie de onde o xiloglucano foi extraído mas, em geral, os oligossacarídeos resultantes possuem quatro glucoses na cadeia principal, três delas com ramificações de xilose, diferindo entre si pelo número e posição das ramificações de galactose (Figura 3) (Vincken et al., 1997). Foi sugerido que esta estrutura básica com um número par de glucoses na cadeia principal seria uma constrição da própria ligação da cadeia principal pois a ligação β (1,4) da cadeia inverte cada resíduo cerca de 180 graus em relação aos resíduos adjacentes. Assim, ao invés de inverter todo o sítio catalítico em relação à cadeia nascente de polissacarídeo, a cada resíduo adicionado, o complexo de biossíntese de xiloglucano adicionaria duas glucoses a cada ciclo de transglicosilação e a unidade básica de biossíntese da cadeia principal seria a celobiose e não a glucose (Carpita e Vergara, 1998).

Embora em solanáceas ocorra uma série de oligossacarídeos com apenas duas xiloses (XXGG), a cadeia principal do oligossacarídeo é ainda assim composta por um número par de glucoses (2 celobioses ligadas, Sims et al., 1996). Em *Hymenaea courbaril* L., no entanto, foi descrita a ocorrência de uma série de oligossacarídeos com 5 glucoses na cadeia principal (Buckeridge et al, 1997). O primeiro membro da série foi purificado e caracterizado como XXXXG e a presença de outros oligossacarídeos foi reportada. Acredita-se que os oligossacarídeos não identificados sejam relacionados ao XXXXG da mesma forma que os oligossacarídeos ortodoxos derivam do XXXG, ou seja, diferem entre si pelo número e posição das ramificações de galactose.

Xiloglucano: Metabolismo de reserva

Biossíntese

A biossíntese de xiloglucano de reserva em sementes ocorre de forma semelhante à biossíntese do xiloglucano de parede primária no Complexo de Golgi. Já foram realizados estudos a respeito da deposição de xiloglucano de reserva em sementes de pelo menos duas espécies: *Tropaeolum majus* e *Tamarindus indica*. Em 1986, Hoth e colaboradores observaram o acúmulo transitório de amido durante a maturação da semente de *Tropaeolum majus* L. (Tropaeolaceae). Este amido desaparecia ao final do processo de deposição do xiloglucano, sendo, provavelmente, utilizado como fonte de carboidratos para a síntese do polissacarídeo de parede. Contudo, a quantidade de xiloglucano formada durante a degradação do amido correspondia apenas a metade desta reserva armazenada, o que sugeriu uma alta atividade em outras vias metabólicas no período (respiração, síntese protéica, etc.). Ao fornecer xilose marcada radioativamente, o amido foi o primeiro polissacarídeo a ser marcado. Segundo os autores, o sistema de biossíntese de xiloglucano não seria capaz de utilizar diretamente xilose livre do citoplasma. A via proposta para o metabolismo da xilose seria a isomerização desta para xilulose, fosforilação e entrada na via das pentoses (xilulose-5-P é um intermediário da via das pentoses). Uma vez no metabolismo primário, a xilulose seria convertida em

glucose-6-P e formaria UDP-glucose. A UDP-glucose seria oxidada a UDP-ácido glucurônico e este seria descarboxilado, formando UDP-xilose que, então, poderia servir de substrato para a biossíntese de xiloglucano.

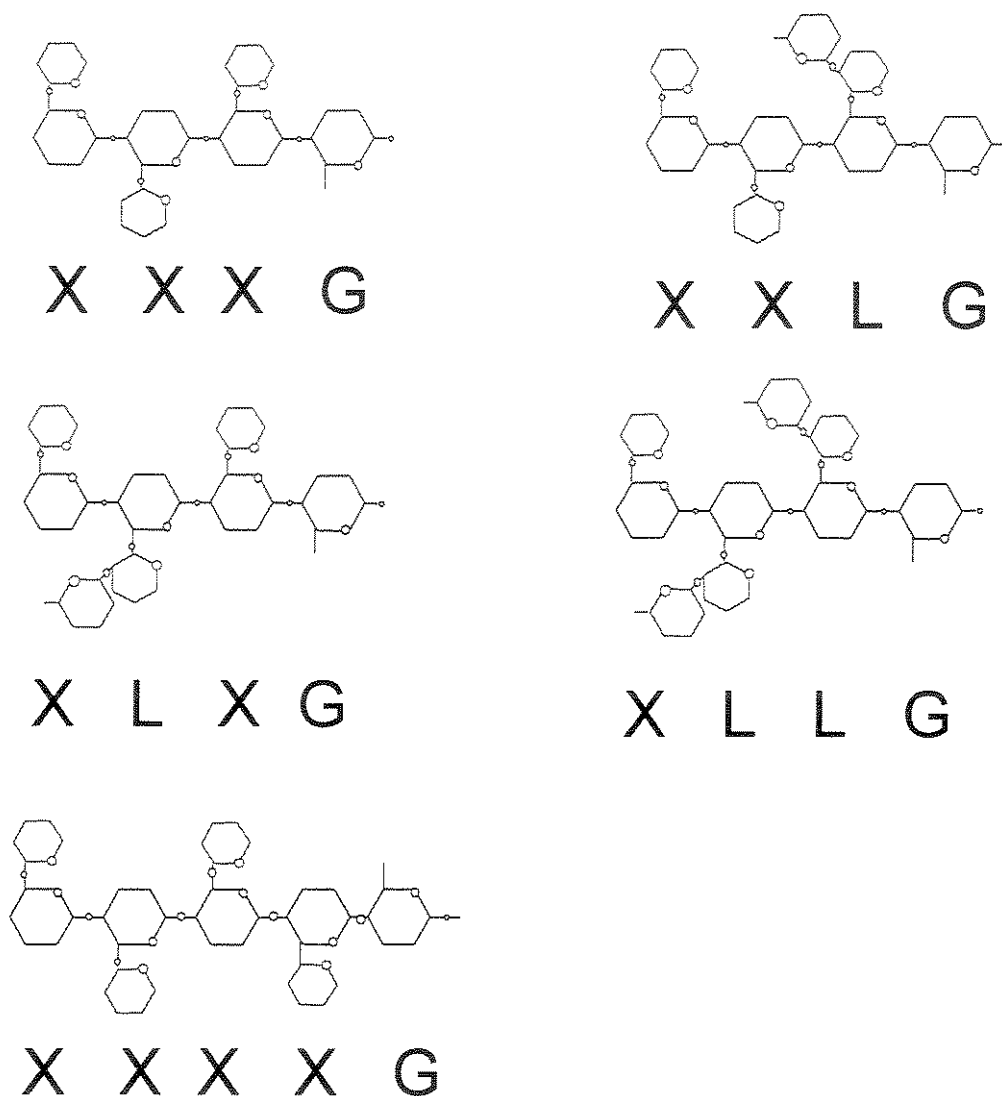


Figura 3. Oligossacarídeos obtidos pela digestão de xiloglucano de reserva com celulase de *Trichoderma reesei*. Os oligossacarídeos com 4 glucoses na cadeia principal são amplamente distribuídos nas plantas (Vincken et al., 1997), mas o XXXXG só foi descrito até hoje em *Hymenaea courbaril* (Buckeridge et al., 1997)

Alguns anos mais tarde, Ruel et al. (1990) acompanharam as alterações na ultraestrutura da parede durante a deposição de xiloglucano nas sementes de *T. majus*. Os autores verificaram que, durante a deposição, havia 3 camadas na parede: a primária, a de reserva e uma terceira camada entre esta última e a membrana plasmática. Esta terceira camada parece ser o sítio de deposição do xiloglucano antes de sua incorporação definitiva à parede. A camada de reserva, por sua vez, apresenta-se heterogênea, rica em alvéolos que, apesar de conterem polissacarídeo, apresentam uma reação menos intensa ao método de impregnação com Iodo que o restante da parede.

Degradação

O sistema de degradação de xiloglucano mais estudado até o momento foi o de *Tropaeolum majus*. Em 1985, Edwards et al. acompanharam a mobilização do xiloglucano nesta espécie e observaram um aumento da atividade das enzimas envolvidas na degradação do mesmo (β -glucosidase, β -galactosidase, α -xilosidase e endo- β 1,4-glucosidase) durante a mobilização deste para o eixo embrionário. Em 1987, Reis et al. acompanharam o mesmo processo em *Tamarindus indica* e observaram o acúmulo de amido no interior da célula durante a degradação do polissacarídeo da parede, supostamente devido ao excesso de carboidrato no citoplasma (como sugerido por Harris e Chrispeels, 1975). Os autores identificaram 3 etapas na mobilização do xiloglucano da semente: uma etapa de hidratação da semente e mobilização dos corpos protéicos, uma etapa de mobilização do xiloglucano e uma etapa pós-mobilização onde o cotilédone permanece ligado ao eixo embrionário com cerca de 10% do peso inicial, sem alterações significativas nas massas de matéria fresca e seca.

Desde então, as principais enzimas de degradação de xiloglucano de *T. majus* foram isoladas e caracterizadas pelo grupo do professor Grant Reid da Universidade de Stirling na Escócia: a β -galactosidase (Edwards et al., 1986; Fanutti et al., 1993), a endo- β 1,4-glucanase (Edwards et al., 1986), a α -xilosidase (Fanutti et al., 1991) e a β -glucosidase (Crombie et al., 1998). Estas enzimas agem em conjunto sobre o polissacarídeo para liberar os monossacarídeos constituintes que seriam internalizados

pela célula. Posteriormente um padrão semelhante de atividades enzimáticas foi encontrado por Alcântara et al. (1999) em *Copaifera langsdorffii*. A β -galactosidase desse sistema foi isolada e, ao contrário da enzima de *T. majus*, ela hidrolisa apenas algumas galactoses em posições específicas, sugerindo a existência de mecanismos sutis e sofisticados de degradação do polissacarídeo (Alcântara et al., 1999). Sabe-se hoje que a endo- β -glucanase presente durante a mobilização do xiloglucano é uma transglicosilase (XET-xiloglucano endo-transglicosilase) (Farkas et al., 1992; Fanutti et al., 1993) membro da família de transglicosidases (Campbel e Braam, 1999) expressa exclusivamente nos cotilédones (Rose et al., 1996).

Embora a presença de xiloglucano de reserva em *Hymenaea courbaril* L. (Leguminosae) já tenha sido sugerida em 1960 (Kooiman, 1960), o acompanhamento da mobilização só foi feito 40 anos depois (Tiné et al., 2000). O acompanhamento da estrutura do polissacarídeo ao longo do processo de mobilização sugeriu a presença de pelo menos duas classes de xiloglucano: uma de reserva (extraível com água quente e pobre em arabinose, cujo teor diminui ao longo do processo) e outra estrutural (extraída apenas com NaOH e rica em arabinose, cujo teor não é alterado ao longo da mobilização das reservas).

Além do acompanhamento citoquímico (Figura 4), a atividade das quatro enzimas de desmonte do xiloglucano foi acompanhada. A caracterização parcial das enzimas levou os autores a propor um mecanismo de controle da degradação baseado no controle da atividade de β -galactosidase pelo pH, de forma análoga ao mecanismo de crescimento ácido induzido por auxina na parede primária (Figura 5). Duas das exo-hidrolases (α -xilosidase e β -glucosidase) possuem um pH ótimo próximo a 4,5, enquanto a outra (β -galactosidase) possui um pH ótimo próximo a 3,5, sendo que em pH 4,5 há uma queda acentuada de atividade. O desmonte do polissacarídeo seria iniciado pela ação de XET sobre o polímero. A caracterização desta enzima mostrou que, ao contrário da XET de *Tropaeolum majus*, a atividade hidrolítica é muito pequena, sendo que difícil detectar a queda da massa molecular na ausência de oligossacarídeos. Após o desmonte do polissacarídeo, os monossacarídeos resultantes seriam internalizados pelas células, convertidos em sacarose e exportados para o eixo embrionário.

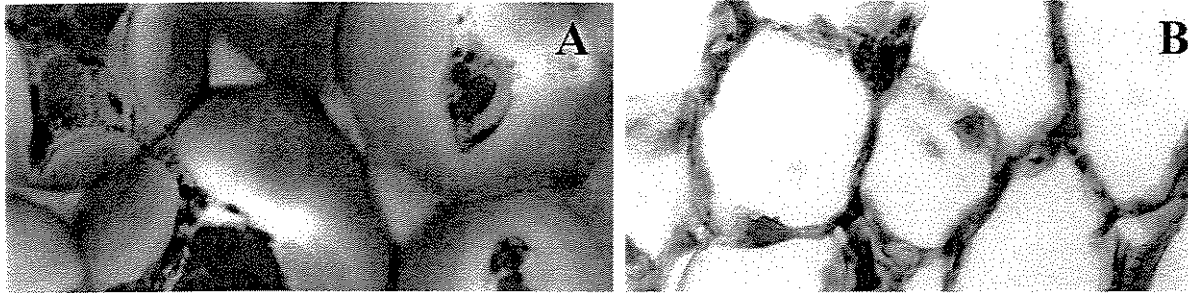


Figura 4. Seções transversais de cotilédones de *H. courbaril* impregnadas com solução de Iodo/Iodeto de Potássio (420x) antes (A) e depois (B) da mobilização do xiloglucano de reserva. O espessamento azul da parede corresponde ao xiloglucano de reserva visto em "A".

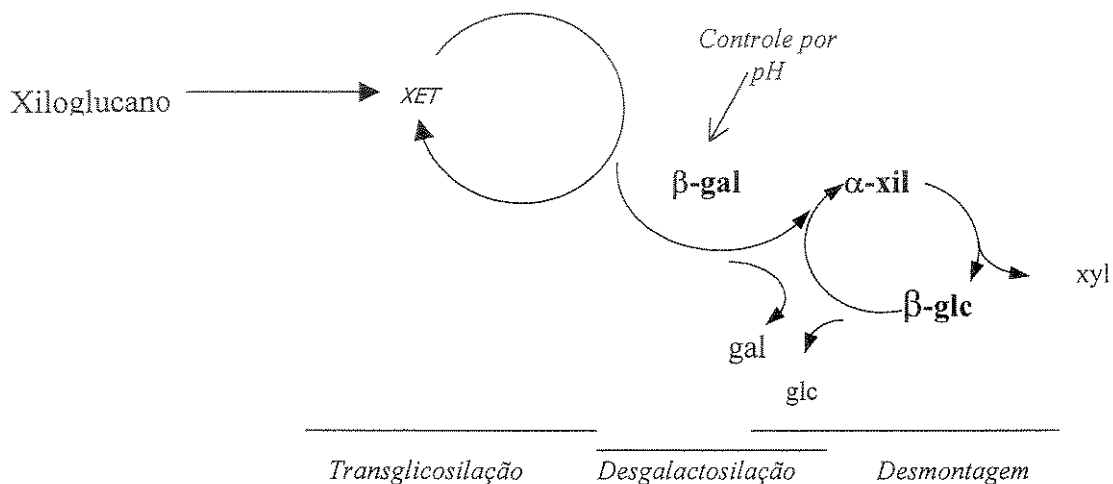


Figura 5. Modelo proposto para a desmontagem "in vivo" do xiloglucano de reserva em sementes. O polissacarídeo é parcialmente hidrolisado pela XET. Somente quando certos resíduos de galactose em XLXG e XLLG seriam hidrolisados (desgalactosilação), os oligossacarídeos remanescentes dos fragmentos maiores contendo XXLG e XXXG entrariam no ciclo final de desmontagem na qual a glucose e a xilose seriam atacadas pela β -glucosidase e α -xilosidase para produzir monossacarídeos livres. XET=xiloglucano endo-transglicosilase, β -gal= β -galactosilase, α -xil= α -xilosidase, β -glc= β -glucosidase. Adaptado de Tiné et al. (2000).

Celulase versus XET

Digestão com celulase tem sido utilizada na caracterização de xiloglucano desde 1980 (Kato e Matsuda, 1980). Estudos estruturais desta enzima mostraram que o sítio catalítico possui entre 0,5 a 1 nm, dependendo do mecanismo da reação (Davies e Henrissat, 1995). No caso das enzimas que mantêm a conformação da ligação (“retaining enzymes”) como a celulase de *Trichoderma resei* vendida comercialmente (Megazyme), o sítio catalítico tem cerca de 0,5 nm, o que corresponde aproximadamente ao comprimento de um resíduo de glucose na cadeia de xiloglucano (Taylor e Atkins, 1985). Esta celulase possui uma estrutura “jelly roll”, com diversos segmentos em folha pregueada formando uma fenda onde a cadeia de substrato se encaixa², colocando o resíduo a ser hidrolisado alinhado com resíduos de ácido glutâmico que agem como doadores e aceptores de elétrons durante a catálise (Figura 6).

As celulasas são inibidas por altas concentrações de produtos como celobiose (Bhat et al., 1989), mas monossacarídeos como a glucose são inibidores bem menos eficientes (Wood e Bhat, 1988). A caracterização do modo de ação das celulasas mostrou que estas enzimas são capazes de transglicosilar (transferir resíduos de açúcares de uma cadeia para outra) (Bhat et al., 1990). O mecanismo da reação deve ocorrer de modo semelhante na hidrólise e na transglicosilação, mas no caso desta última reação, oceptor da cadeia é a hidroxila de um outro carboidrato, e não a água. A transglicosilação em celulase só ocorre em condições de alta concentração de produto ou em soluções etanólicas, mas o fato de ser possível força-la em enzimas hidrolíticas levanta importantes questões sobre a origem evolutiva da xiloglucano endo-transglicosilase (XET), a enzima de plantas responsável pela hidrólise “endo” da cadeia de xiloglucano. Uma delas é se estas enzimas teriam um ancestral em comum.

² Informações atualizadas sobre esta classe de enzimas estão disponíveis no site <http://afmb.cnrs-mrs.fr/CAZY>

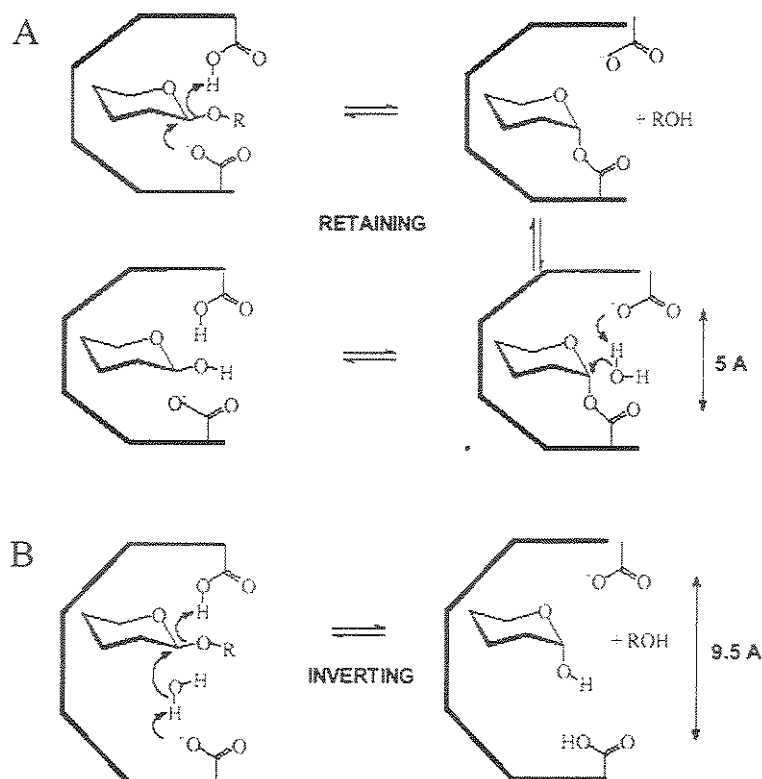


Figura 6. Mecanismos de hidrólise de ligações glicosídicas. A reação de hidrólise de ligação pode manter a posição da hidroxila anomérica (A-retaining) ou inverter a posição (B-inverting). Tanto na XET quanto na celulase de *Trichoderma reesei*, os resíduos ácidos são ácidos glutâmicos. O sítio catalítico das enzimas “inverting” é maior para poder acomodar uma molécula de água para efetuar hidrólise em um único passo. Adaptado de Davies e Henrissat (1995).

A comparação da estrutura primária das XETs e outras enzimas hidrolíticas coloca a XET mais próxima a outras hidrolases como liquenase ($\beta(1,4),(1,3)$ glucosil hidrolase) e a $\beta(1,3)$ glucanase, mas todas estas enzimas são estruturalmente muito próximas à celulase, possuindo a estrutura básica “jelly roll”, com folhas pregueadas β formando uma superfície onde o substrato ancora. Nenhuma XET, no entanto, teve sua estrutura terciária determinada por cristalografia ou Ressonância Magnética Nuclear até o momento, de forma que estas asserções a respeito da estrutura terciária são feitas apenas por semelhança entre as estruturas primárias. Estas semelhanças reforçam a idéia de ser possível comparar os dados relativos ao modo de ação destas enzimas, embora

elas sejam obtidas de origens muito distintas (celulase fúngica e XET de plantas). As diferenças entre estas duas enzimas provavelmente se devem às diferentes funções para os organismos que as produzem. Plantas produzem celulases apenas em algumas situações muito específicas como formação de aerênquima, por exemplo. A enzima mais amplamente distribuída em tecidos vegetais que age sobre os β -glucanos são transglicosilases (xiloglucano endo-transglicosilase-XET). A transglicosilação realizada pela XET, provavelmente está ligada ao metabolismo do xiloglucano na planta, permitindo o crescimento controlado da célula, enquanto a hidrolase produzida por microorganismos estaria ligada ao processo de invasão e degradação da parede vegetal.

A análise da estrutura fina do xiloglucano de sementes de *Tropaeolum majus* L., *Tamarindus indica* L. e *Copaifera langsdorffii* L. com XET de *Tropaeolum majus* L. mostraram que esta enzima libera como produtos de reação os mesmos oligossacarídeos liberados pela ação da celulase de *Trichoderma*: XLLG, XXLG, XXXL e XLLG, sugerindo uma semelhança no modo de reconhecimento do substrato na celulase e na XET. Até o momento, as únicas XETs purificadas a partir de sementes que acumulam xiloglucano como reserva foram as de *T. majus* (Edwards et al., 1986 e Fanutti et al., 1993) e de *Hymenaea courbaril* (Alcânrra, 2000). A caracterização da primeira enzima mostrou que ela requer uma série de 3 glucoses da cadeia principal para poder reconhecer o substrato. O resíduo cuja ligação vai ser quebrada não pode estar ramificado, mas como a ligação da cadeia inverte os resíduos adjacentes, estes dois podem apresentar ramificações. A XET de *H. courbaril*, ao contrário da enzima de *T. majus*, só é capaz de reduzir o tamanho do xiloglucano na presença de oligossacarídeos, o que significa que esta enzima apresenta uma capacidade hidrolítica muito limitada (Tin   et al., 2000), mesmo em baixas concentra  es de xiloglucano e praticamente s      capaz de transglicosilar. A XET de *T. majus*, por outro lado,    capaz de produzir oligossacar  deos em baixas concentra  es de substrato e apenas quando a concentra  o de fragmentos de xiloglucano (receptores da transglicosila  o) aumenta, a transglicosila  o passa a ser a rea  o predominante. Diante das semelhan  as apontadas entre a XET e a celulase, acreditamos que as informa  es obtidas por hidr  lise com celulase f  ngica possam ser estendidas ao metabolismo da parede.

Justificativa

Todas as plantas apresentam uma etapa heterotrófica no seu ciclo de vida. Na maioria das Angiospermas, a semente é uma das etapas mais vulneráveis do ciclo de vida, pois representa um momento em que o novo indivíduo ainda não é capaz de extrair do ambiente todos os recursos necessários à sua sobrevivência. A diversidade de nossa flora e a diversidade de estratégias de crescimento acaba sendo refletida, em certo grau, na diversidade de compostos de reserva das espécies tropicais. Apenas entre os carboidratos poliméricos, podemos citar plantas que acumulam amido, frutanos, (galacto)mananos e xiloglucanos. Nesta etapa em que os indivíduos são heterotróficos e dependem das reservas provenientes da planta mãe, o metabolismo dos carboidratos de reserva é uma das peças-chave na estratégia de vida da planta e sua distribuição.

Dentre as sementes que acumulam xiloglucano, apenas quatro espécies tiveram sua estrutura fina (composição de oligossacarídeos) estudada até o momento:

Tropaeolum majus, *Tamarindus indica*, *Copaifera langsdorffii* e *Hymenaea courbaril*.

As três últimas pertencem ao mesmo grupo taxonômico (*Leguminosae*,

Caesalpinioideae, tribo Detarie), enquanto *T. majus* é uma *Tropaeolaceae*. De fato, as

leguminosas se prestam muito bem a estudos da evolução da reserva de carboidratos,

pois dentro da família é possível encontrar as mais diferentes estratégias de

sobrevivência e de metabolismo de carboidratos de reserva. É possível encontrar desde

sementes que possuem grandes quantidades de reserva de carboidrato como *H.*

courbaril até sementes com baixos conteúdos de reservas como *Piptadenia*

gonoacantha. Ainda dentro da família existe uma grande diversidade de polissacarídeos

de reserva que ocorrem em sementes: amido, galactano, (galacto)manano e xiloglucano

(Buckeridge et al., 2000a).

Embora Lima e colaboradores (1993) tenham definido a estrutura do xiloglucano de *H. courbaril* como sendo um xiloglucano “ortodoxo” (ou seja, formado basicamente por repetições de blocos de quatro glucoses, como o xiloglucano de *Tamarindus indica* e *Tropaeolus majus*), Buckeridge et al. (1997) revelaram a existência de uma nova classe de oligossacarídeos gerados por digestão com celulase. Cerca de metade do

xiloglucano de reserva de *H. courbaril* é composta por um polissacarídeo baseado em oligossacarídeos formados por 5 glucoses, ao contrário das estruturas já descritas, formadas basicamente por grupos de 4 glucoses.

O jatobá (*Hymenaea courbaril*) é uma leguminosa arbórea que tem sido explorada comercialmente por fornecer uma madeira dura e resistente à putrefação, que pode ser usada em construções pesadas e obras hidráulicas (Carvalho, 1994). Como uma espécie típica de estágios avançados de sucessão em florestas neotropicais, o jatobá é uma planta bastante exigente quanto às condições de crescimento e desenvolvimento. Com a redução das áreas de mata nativa do país, esta espécie, assim como várias outras, correm o risco de entrar em extinção. Estudos a respeito de sementes de espécies tropicais são, portanto, um passo importante para desenvolver estratégias de manejo e conservação de florestas onde estas ocorrem (Janzen e Vázquez-Yanes, 1991).

Um outro aspecto importante a ser salientado é que apesar das diferenças nas proporções de monossacarídeos entre o xiloglucano de parede primária e o de reserva, este último é um bom modelo de estudo de polissacarídeo de parede. A sua abundância facilita a obtenção de material para estudos, e por requerer uma grande quantidade de enzimas para sua hidrólise, facilita também a obtenção destas para uso como ferramentas no estudo dos polissacarídeos. Além disso, a existência de um espessamento rico em xiloglucano levanta outras perguntas a respeito da capacidade de auto-interação deste polissacarídeo, pois nos modelos de parede existentes, o xiloglucano está sempre associado à celulose e entremeado por outros polímeros, enquanto nos casos de paredes de reserva a celulose nunca foi detectada.

Objetivos

O presente trabalho teve como objetivos caracterizar os padrões de ramificação do xiloglucano de reserva de *Hymenaea courbaril* e avaliar a influência destas cadeias laterais na interação com celulase em comparação com os xiloglucano de reserva de outras espécies que não possuem oligossacarídeos com cinco glucoses em sua cadeia. Como as cadeias laterais podem modular a interação entre a enzima e o substrato, um

novo padrão de ramificação no xiloglucano pode ter efeitos no catabolismo do polissacarídeo.

Este estudo também visa imunolocalizar o xiloglucano de reserva na parede celular. Da mesma forma que nos estudos bioquímicos, serão utilizadas outras espécies que acumulam xiloglucano em suas sementes para comparação.

Artigo 1

**Structure of three new galactosylated oligosaccharides of the
XXXXG family from the storage xyloglucan of *Hymenaea courbaril* L.**

A ser submetido à revista “Carbohydrate Research”.

Structure of three new galactosylated oligosaccharides of the XXXXG family from the storage xyloglucan of *Hymenaea courbaril* L.

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Running title: Galactosylated oligosaccharides from the XXXXG family

abbreviations:

HPAEC-PAD, High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection; ESI-MS, Electrospray ionisation -Mass Spectrometry;

Keywords: Xyloglucan, *Hymenaea courbaril* L., storage, polysaccharides, cell wall, electrospray-mass spectrometry.

Abstract

Three new oligosaccharides from the storage xyloglucan of *Hymenaea courbaril* were purified by size exclusion chromatography. The proportion of monosaccharides obtained by acid hydrolysis for each of these three oligomers was 5:4:1 (glucose:xylose:galactose), indicating that they differ from each other only by the position of a single galactosyl residue. Of the three oligosaccharides, only one was susceptible to *Copaifera langsdorffii* β -galactosidase to generate XXXXG and was identified as XLXXG. All three oligosaccharides had the same mass to charge ratio as determined by mass spectrometry analysis ($m/z=1541$), but produced different secondary fragments, after ESI-MS. Only one oligomer produced "LG" fragment ($m/z=659$), characteristic of XXXLG, and one produced "XL" fragment ($m/z=773$), indicative of XLXXG and consistent with the susceptibility to β -galactosidase hydrolysis. The third oligosaccharide did not produce any specific fragment that could only be generated by a specific oligosaccharide, but had ESI-MS profiles consistent with XXLXG. These results allowed us to identify the three oligosaccharides as XXXLG, XLXXG and XXLXG. Thus, unlike other xyloglucans described in the literature, *H. courbaril* storage xyloglucan is composed of five glucose units, in addition to the four-glucose units normally found. The ESI-MS of dimers of oligosaccharides detected the preferential presence of the two series in the same polysaccharide, suggesting that they are alternated in the polymer, with implications in the distribution of the galactoses along xyloglucan chain. This imposes a re-evaluation of the strict cellobiosyl/cellotetraosyl unit structure, indicating a novel synthesis mechanism.

Introduction

Xyloglucan is a major matrix glycan in the primary walls of all dicots and several monocot species. Some seeds store this polysaccharide in large amounts as a resource for the embryo after germination (Buckeridge et al., 2000). The seed storage xyloglucan has a cellulosic-like (1→4) β- D-glucan backbone, where some glucose residues are substituted with (1→6)α- D-xylopyranoside and these can be further substituted with (1→2)β-D-galactopyranoside. According to the current nomenclature (Fry et al., 1993), the unbranched glucose residues are named “G”, the xylose-substituted glucoses are named “X” and the trisaccharide formed by glucosyl with xylosyl and galactosyl attached are designated “L”. This basic structure is shared by all xyloglucans described to date, but in the primary wall, further substitutions with fucose and arabinose are common (Hayashi, 1989).

The most widespread technique used for studies on xyloglucan has been the digestion with a *Trichoderma* endo-β-glucanase (cellulase). This enzyme hydrolyses xyloglucan polymers only at glycosidic linkages where an unbranched glucose residue is present. This pattern of action produces a few structurally distinct oligosaccharides that usually have four glucoses in the main chain, differing from each other by the position and length of the side-chains (Vincken et al., 1997a). This basic structure was suggested many years ago (Kooiman, 1960) from the proportions of monosaccharides. It was assumed that this four-glucoses structure was a restriction imposed by the inversion of the glycosyl residues typical of (1,4)-β linkages (Carpita and Vergara, 1998). Since xyloglucan and cellulose share the same glycosyl backbone, it was proposed that cellobiose was the basic unit of both polysaccharides.

One of the few exceptions in the XXXG-based xyloglucan is the polysaccharide from Solanaceae species, where only two contiguous glucose residues are substituted with xylose (Sims et al., 1996). But even in this case, the third glucosyl residue is acetylated at position 6, and the basic structure is also composed of a cellotetraosyl backbone (XXGG).

The storage xyloglucan from *Hymenaea courbaril* L., on the other hand, was the only xyloglucan to date shown to contain a 1:1 proportion of a 5-glucose backbone oligosaccharides, besides the usual family of XXXG-based oligosaccharides (Buckeridge et al., 1997). The first oligosaccharide of this series to be purified and characterised was XXXXG, but the authors pointed out to the existence of other unidentified oligosaccharides produced by cellulase from polymeric xyloglucan and suggested the presence of a family of galactosylated oligosaccharides with five glucoses in the main chain. In this work, we show that three of these oligosaccharides are produced by the addition of a single galactosyl residues at the three possible positions of attachment. Electrospray Mass Spectrometry analysis of fragments containing two constitutive blocks of *H. courbaril* xyloglucan revealed that the polymer is formed by alternation of XXXG and XXXXG. The implications of our discoveries for the mechanism of xyloglucan biosynthesis and mobilisation are discussed.

Results

Isolation and linkage analysis

Three unidentified oligosaccharides were purified from *Hymenaea courbaril* seed storage xyloglucan by size exclusion chromatography on Biogel P-4 and named Y1, Y2 and Y3, according to their elution profile in the HPAEC-PAD (Figure 1). These three oligosaccharides represent approximately 30% of the total oligosaccharides produced by the action of cellulase on the polymer (Table 1). The linkage analysis of each purified oligomer showed the expected linkages (4,6-glc:6-glc:4-glc) for a storage xyloglucan oligosaccharide. The ratio between the glucose linkages in the oligosaccharides (Table 2) was approximately 3:1:1 (4,6-glc:6-glc:4-glc), confirming the initial hypothesis that these oligosaccharides have 3 branched glucoses in the middle of its structure for each reducing and non-reducing end. No arabinose or fucose were detected. The proportion of monosaccharides determined by acid hydrolysis and analysis by HPAEC-PAD was the same for all three oligosaccharides (5:4:1 glc:xyl:gal), indicating that they differ from each other only by the position of one galactosyl

residue as side chain. Methylation analysis of the polymeric xyloglucan did not show the presence of any other glycosidic linkage regarding galactose apart from the typical 1,2- β (See paper 3). 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 or XXXLG. However, the position at which galactosyl is linked in each oligosaccharide could not be deduced from their order of elution in the HPAEC-PAD.

Digestion of xyloglucan oligosaccharides with β -galactosidase

In order to deduce the position of galactose in at least one of the oligosaccharides, we used β -galactosidase from cotyledons of *Copaifera langsdorffii*, which has been demonstrated to hydrolyse galactose only at the "terminal" position (XL--) of xyloglucan oligosaccharides (Alcântara et al., 1999). Of the three possible structures, only one of them (XLXXG) should be susceptible to the action of *C. langsdorffii* β -galactosidase. The hydrolysis confirmed this hypothesis as only one of the oligosaccharides ("Y2") was susceptible to the action of the enzyme, producing XXXXG (Figure 2). The oligosaccharide Y2, therefore, was identified as XLXXG.

Analysis of purified oligosaccharides and limited digest polymers by electrospray mass spectrometry

Figure 3 shows the electrospray mass spectrometry analyses of the mixtures of cellulase limit digest oligosaccharides from *Tamarindus indica* and *H. courbaril*. In *T. indica* the presence of fragments m/z 1085, 1247 and 1409 are consistent with the presence of XXXG, XLXG/XXLG and XLLG, respectively. The *H. courbaril* storage xyloglucan contained much less of the ion $m/z=1085$ (XXXG), large amounts of ion $m/z=1247$ (mainly XXLG, according to HPAEC-PAD analysis) and a limited amount of the ion $m/z=1409$ (XLLG). The XXXXG series is clearly superposed to the XXXG oligosaccharides as can be deduced by the presence of XXXXG ($m/z=1379$), XLXXG/XXLXG/XXXLG ($m/z=1541$) and XLLXG/XLXLG/XXLLG ($m/z=1703$).

The analysis of the purified oligosaccharides showed that all of them had the same ratio mass/charge ($m/z=1541$). The secondary fragmentation of this ion, however, produced different fragments that allowed the identification of the position of the galactosyl residue in each oligosaccharide (Figure 4). One of the fragments produced only by Y1 was the one assigned to “LG” ($m/z=659$). Since xyloglucan oligosaccharides always have an unbranched glucose at the reducing end as a result of the mode of action of the fungal cellulase on the polymer, the only oligosaccharide of the three possibilities that could produce such a fragment was XXXLG. The oligosaccharide Y2 produced a fragment assigned to “XL” ($m/z=773$), confirming our enzymatic identification of Y2 as being XLXXG. The oligosaccharide Y3 did not produce any fragment that could only be generated by specific oligosaccharides, but the fragmentation obtained was compatible with XXLXG, the only possibility that fit the data (Figure 4).

The ESI-MS of the mixture of oligosaccharides in the dimers range (around m/z 2500) showed several ions (Figure 5). The dimers XXXGXXXXG ($m/z=2423$), XLGXXXXG ($m/z=2585$), XLGXLLG ($m/z=2615$) and XLLGXXXXG ($m/z=2747$) were identified, indicating that both series of oligosaccharides are present in the same xyloglucan molecule and arranged predominantly in an alternated fashion. The ion $m/z=2585$ can also be assigned to the oligosaccharides XXXGXLXXG, XXXGXXLXG or XXXGXXXLG, since all of them have the same molecular weight.

Discussion

Oligosaccharides characterisation- the results presented in this work fully confirm that *Hymenaea courbaril* synthesises a storage xyloglucan with unique structural features, such as an elongated branched main chain composed of 5-glucose oligosaccharides and also that it has a singular pattern of galactose branching throughout the polymer. Results from our laboratory showing that the storage unfucosylated wall is present among two primaries fucosylated inner and outer walls (see paper 3) suggest that the XXXXG based polymer appears to have exclusively storage function.

The three most abundant unidentified oligosaccharides from *Hymenaea courbaril* storage xyloglucan were isolated by size exclusion chromatography and identified by ESI-MS. All of them had only one galactose and differ from each other only by the position of the galactosyl side chain. As expected from the monosaccharide analysis, the mass/charge ratio of the oligosaccharides was the same ($m/z=1541$), but the ESI-MS of the purified fractions allowed the analysis of the specific fragmentation for each oligosaccharide. The first two oligosaccharides produced diagnostic fragments that made possible the precise identification as XXXLG and XLXXG. The fragmentation of the third oligosaccharide was compatible with the only possibility left from the monosaccharide analysis (XXLXG). Furthermore, the ratio between the glucose linkages indicates that there are three glucoses in middle of the oligosaccharide chain (4,6-linked glc) for each reducing (4-linked glc) and non-reducing end (6-linked glc).

Xyloglucan biosynthesis- although no model for the biosynthesis of xyloglucan has been proposed to date, Hayashi (1981) suggested that the glucosyl- and the xylosyl-transferases act on the “cis” face of the Golgi complex, assembling the glucose-xylose backbone. The mechanism of interaction between these two transferases is still unclear, but the models proposed from now on will have to take into account both families of oligosaccharides (with 4 and 5 glucoses in the backbone). The existence of two different domains in the wall of the storage cells with two different xyloglucans (fucosylated and non-fucosylated) (see paper 3) indicates that the cotyledons of *H. courbaril* have at least two classes of xyloglucan: the structural and the storage (as suggested by Tiné et al., 2000). During seed maturation, the shift from the structural to the storage xyloglucan in the parenchyma cells possibly involves changes in the biosynthetic complex, probably with shift in the expression of different glucosyl and xylosyl-transferase genes. The existence of functionally and structurally different xyloglucans could explain the need of families of transferases in the plant (Carpita and Vergara, 1998), since the biosynthesis of different polymers could require different genes for the transferases.

The amount of oligosaccharides with galactose adjacent to the non-reducing end (“XL...” oligosaccharides) is approximately 24% in *H. courbaril* (Table 2). In fact, the amount of galactose released by the β -galactosidase when the total mixture of

oligosaccharides is used as substrate is negligible (data not shown), especially when compared with *C. langsdorffii* mixture, which is richer in “XL...motifs. This indicates that xyloglucan disassembling mechanism in *H. courbaril* is slightly different from other xyloglucan-storing seeds. Once the oligosaccharide is released by XET in the apoplast, the first enzyme that acts upon it is the α -xylosidase. Since β -galactosidase seems to be an important step in the control of degradation in species other than *Hymenaea*, some differences in the control of xyloglucan disassembling may apply to this species. The unusual branching pattern could also lead to different kinetics of interaction of XET with the polysaccharide, as happens with cellulase (Vincken et al, 1997a). If we consider the effect of the side chains in the recognition of the substrate by the enzyme, this change in xylosil branching pattern means that part of the information required to the disassembling process resides on the substrate itself and is “programmed” during xyloglucan deposition.

Almost all dimers of oligosaccharides identified in the ESI-MS were mixtures of oligosaccharides from the two series (with 4 and 5 glucoses) indicating a tendency for alternation of the two series of oligosaccharides along the polymer. Therefore, the use of the nucleotide-sugars during xyloglucan biosynthesis is strictly controlled by the enzymes and the proportion of glucose and xylose used probably does not depend only on the availability of the substrate and the random occupation of the active sites. This alternation also has implications in the distribution of the galactoses along the main chain. The fifth glucose inverts the chain 180°, therefore 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 but richer in XXLG, like the one of *H. courbaril*. These two alternatives (enrichment in XLLG and presence of XXXXG) seem to be two options to cope with the same problem: how to assemble a functional wall with a proportion of cellulose and hemicellulose so different from the primary wall.

Implications to xyloglucan disassembling- the activity of the *C. langsdorffii* β -galactosidase on XLXXG confirmed that the enzyme has high specificity to “XL” motives positioned at the non-reducing end of xyloglucan oligosaccharides. Despite the

lack of kinetic studies on the influence of the length of the oligosaccharide on the activity of this enzyme, the substitutions or length of the oligosaccharide chain at the reducing end seem to have little or no effect on the recognition of the substrate, since the mode of action of this enzyme upon the 5-glucose oligosaccharides was the same as upon the 4-glucose oligosaccharide. This would be expected from an enzyme involved in xyloglucan degradation, since in each cycle of oligosaccharide disassembling with α -xylosidase and β -glucosidase (Tin   et al., 2000), the oligosaccharide would become one glucosyl residue closer to the reducing end, but the next possible position of the galactosyl would still be at the same distance from the non-reducing end.

The storage xyloglucan of *H. courbaril* differ from other storage xyloglucans (*Copaifera langsdorffii*, *Tropaeolum majus* and *Tamarindus indica*) in at least two points: it has oligosaccharides with one more isoprimeverose unit and the amount of galactosyl close to the non-reducing end ("XL...") is lower. In *Tropaeolum majus*, for example, the presence of XLLG can be used as a marker of storage xyloglucan deposition (Desveaux et al., 1998). The galactosyl transferase detected in pea microsomes (i.e. primary cell wall) add galactosyl residues preferentially in the first xyloxyl residue from the reducing end of the oligosaccharide, producing more XXLG than XLXG (Faik et al., 1997). The abundance of XLLG in storage xyloglucan from most species suggest the existence of more than one galactosyl transferases, each with a different affinity for their substrate. On the other hand, in *H. courbaril*, the change in the biosynthesis pattern seem to be rather related to a change in xylosyl transferase.

Which properties of the polysaccharide would favour the establishment of a new structure on *H. courbaril*? The impact of these structural features on the physicochemical properties of the storage xyloglucan is not clear yet, but the presence of this fifth glucose in the main chain inverts the position of the side chains and creates a unique pattern of xylosyl and galatosyl side chain, without changes in the proportion of the monosaccharides (Buckeridge et al., 1997) and, in principle, without an increase in the energetic costs to produce the polymer. With large seeds, which accumulate approximately 2 grams of xyloglucan per seed, a possible explanation may be linked to the packing of the wall or mechanisms of control of degradation. In these storage walls,

there is little or no cellulose and xyloglucan has to interact with other xyloglucan molecules to form a solid matrix. Changes in the branching pattern could have a profound influence on the interaction of these molecules. Apparently, the two groups of xyloglucan-storing species have solved the problem in two different ways: one group with the xyloxy transferase (*H. courbaril*) and the other with the galactosyl transferase (*C. langsdorffii*, *T. indica* and *T. majus*).

Material and methods

Isolation of β -galactosidase from cotyledons of *Copaifera langsdorffii*

Seeds of *C. langsdorffii* were collected from trees cultivated at the Instituto de Botânica de São Paulo and stored at 5°C. Seeds were soaked in water and after full imbibition they were planted in pots containing a mixture of forest soil and sand (1:1). Enzyme preparation followed exactly the same procedure used by Alcântara et al. (1999), resulting in a single polypeptide band. No residual activities of either α -xylosidase or β -glucosidase were present after checking by assaying these enzymes directly or after HPAEC analysis of the products of hydrolysis of a mixture of xyloglucan oligosaccharides previously incubated with pure β -galactosidase.

Purification of the oligosaccharides

Xyloglucan was prepared from the powder of seeds of *Hymenaea courbaril* as described by Buckridge et al. (1997). Five mL of a 0.5% (w/v) solution of xyloglucan in Na⁺ Acetate buffer (50 mM pH 5,0) were digested with 200 μ L of a 1:100 dilution of *Trichoderma viride* cellulase (Megazyme, 500 U/mL in 3.2 M ammonium sulfate, pH 7) for 24 hours at 30°C. The solution of oligosaccharides produced was loaded onto a Biogel P-4 (BioRad) column (1,5 x 170 cm) and eluted with a 0.05% solution of NaN₃. Fractions of 1 mL were collected and carbohydrates were quantitatively analysed by Phenol-Sulfuric method (Dubois et al., 1956). The qualitative analysis of the fractions were performed by High Performance anion Exchange Chromatography with Pulsed

Amperometric Detection (HPAEC-PAD) with a CarboPak PA-100 column (Dionex). The separation was performed in 88mM NaOH (constant) with a linear gradient of sodium acetate from 35 to 75 mM for 20 minutes followed by another linear gradient from 75 to 200 mM until 50 minutes ($0.9 \text{ mL} \cdot \text{min}^{-1}$). Fractions that contained the same oligosaccharide were pooled.

Monosaccharide analysis

Volumes corresponding to 50 μg of each purified oligosaccharide were freeze-dried in glass vials and dissolved in 350 μL of 1N HCl. The tubes were sealed and heated at 100°C for two hours. The monosaccharides produced were analysed by HPAEC-PAD in a CarboPak PA-1 column (Dionex) with 20 mM NaOH for 40 minutes ($0.8 \text{ mL} \cdot \text{min}^{-1}$).

Digestion with β -galactosidase

The β -galactosidase from *Copaiifera langdorffii* was purified according to Alcântara (1999). This enzyme can only remove the galactose linked to the second xylose from the reducing end of xyloglucan oligosaccharides, therefore, it recognises as substrate only the "XL" in the non-reducing end of the oligosaccharide, but not "XXL". A hundred microliters of fractions from Biogel P-4 column were digested with 20 μL of β -galactosidase ($480 \text{ } \mu\text{mol gal} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$ upon pNP- β -D-galactopyranoside) at 30°C for 24 h. The sample was directly loaded in the liquid chromatograph (HPAEC-PAD) for analysis of oligosaccharides.

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 (1993). Partly methylated alditol acetates were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry as described by Carpita and Shea (1989).

Electrospray MS and MS/MS

The samples were analysed by electrospray MS on a Finnigan MAT liquid chromatograph quadrupole mass spectrometer system (Thermoquest Corp., San Jose, CA). Freeze-dried samples containing 0.1 to 1 mg of digest 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 per min, and the drying gas was nitrogen. The samples were typically scanned from m/z 300 to m/z 2000. 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 (MS/MS) experiments. The collision energy was set at 40 to 50% of a maximum of 5 V “tickle” voltage, depending on abundance and mass of the fragment. Gramicidin, m/z 570.7, was used for mass calibration, and calculations of m/z for all molecular ions and their fragments were within experimental error limits of 0.06%, as established for electrospray MS by Haynes et al. (1997).

Acknowledgments

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Table 1. Proportion (in percentage of the peak areas) of limit digest oligosaccharides obtained by HPAEC-PAD in the three legumes where storage xyloglucan has been analysed. The last line ("other") refers to the mixture of oligosaccharides with two and three galactosyl residues that were detected but not identified.

Oligosaccharides	<i>C. langsdorffii</i>	<i>T. indica</i>	<i>H. courbaril</i>
XXXG	12.5	24.4	3.2
XLXG	10.8	8.0	traces
XXLG	25.7	36.0	25.6
XLLG	51.0	31.6	13.1
XXXXG	-	-	11.7
XXXLG (Y1)	-	-	12.4
XLXXG (Y2)	-	-	11.2
XXLXG (Y3)	-	-	4.9
Other	-	-	18.0

Table 2. Proportion between glucose linkages in the purified fractions of oligosaccharides.

	<i>4-linked glc</i>	<i>6-linked glc</i>	<i>4,6-linked glc</i>
XXXLG (Y1)	1.06	1.56	3
XLXXG (Y2)	0.85	1.33	3
XXLXG (Y3)	0.63	1.10	3

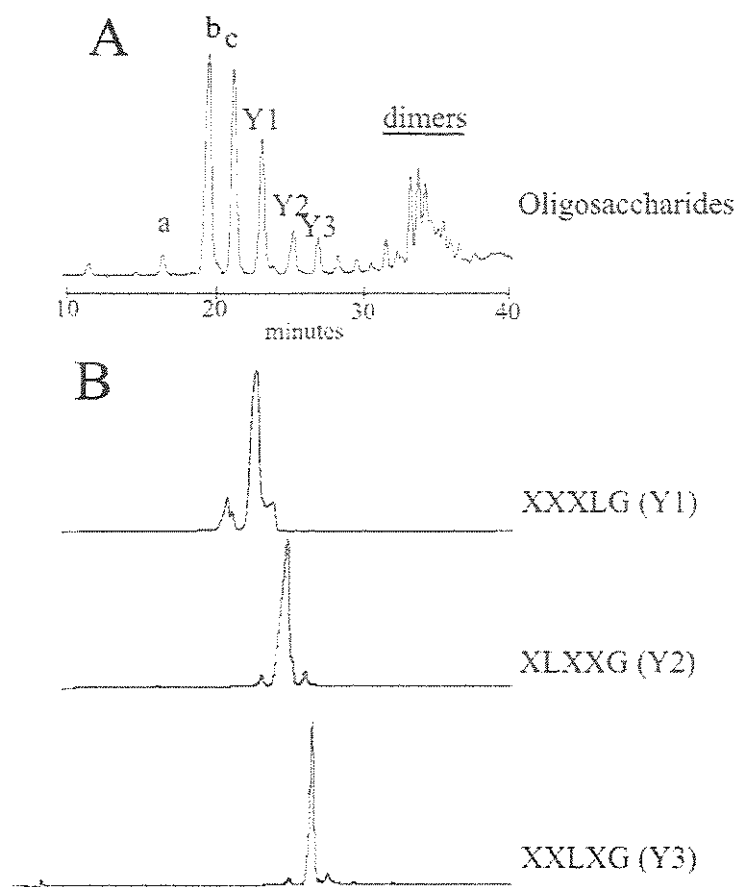


Figure 1. Analysis by HPAEC-PAD of the oligosaccharides obtained from *H. courbaril* storage xyloglucan after hydrolysis with cellulase. A- oligosaccharides mixture showing the presence of the four and five glucose series of oligosaccharides in the total cellulase limit digest. B-oligosaccharides purified by gel filtration on Biogel P-4. The oligosaccharides were labelled according to their order of elution from the column: Y1, Y2 and Y3. The known oligosaccharides are: a-XXXG, b-XXLG and c-mixture of XLLG and XXXXG.

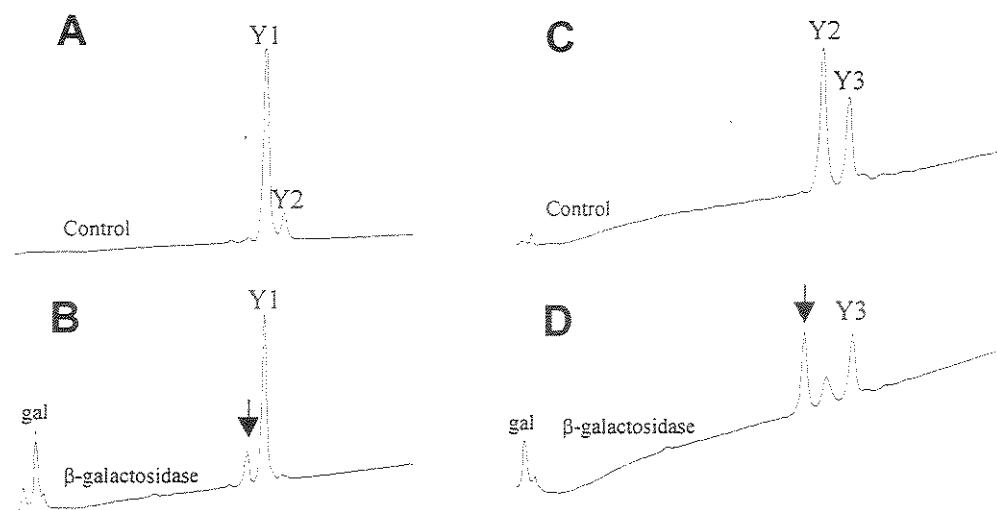


Figure 2. Digestion of the oligosaccharides with *C. langsdorffii* β -galactosidase. Biogel P-4 fractions (A and C) were hydrolysed with β -galactosidase (B and D). The oligosaccharides Y1 and Y3 were not attacked by the enzyme, while Y2 generated XXXXG (arrow) and free galactose (gal).

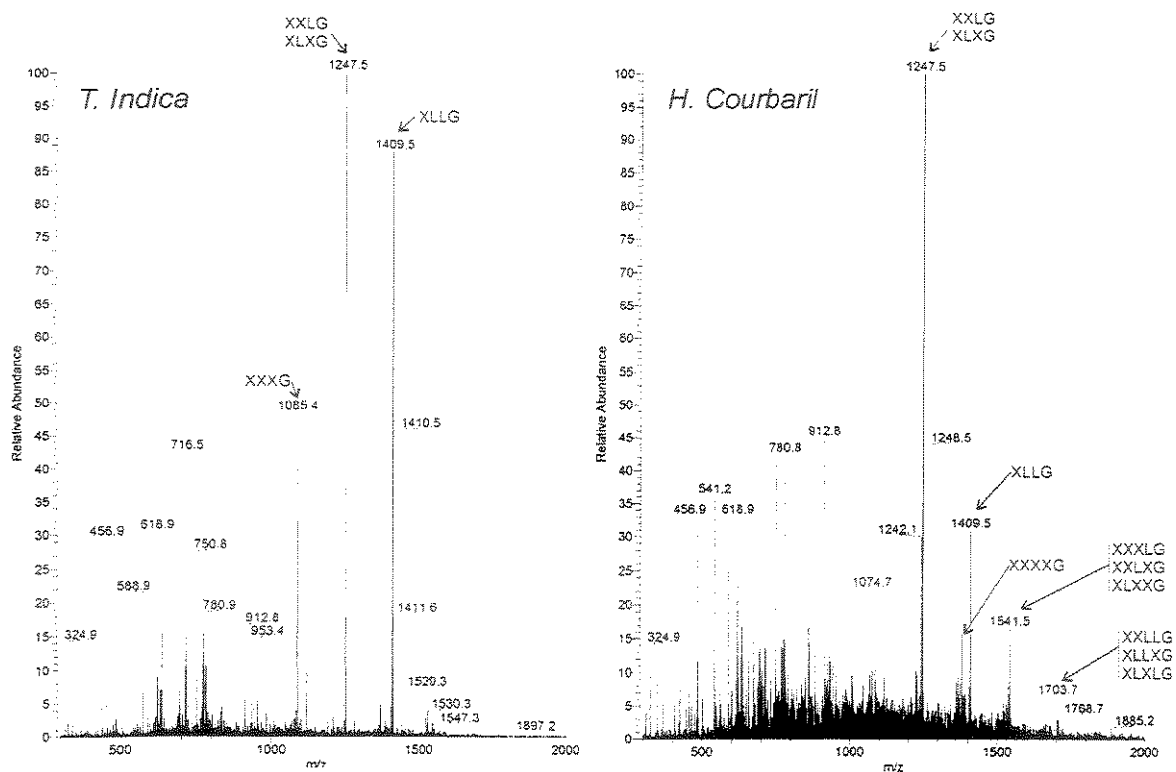


Figure 3. Electrospray mass spectrometry (ESI-MS) of storage xyloglucan from *Tamarindus indica* and *Hymenaea courbaril*. The polysaccharide from *T. indica* does not contain the 5-glucose oligosaccharides present in *H. courbaril*.

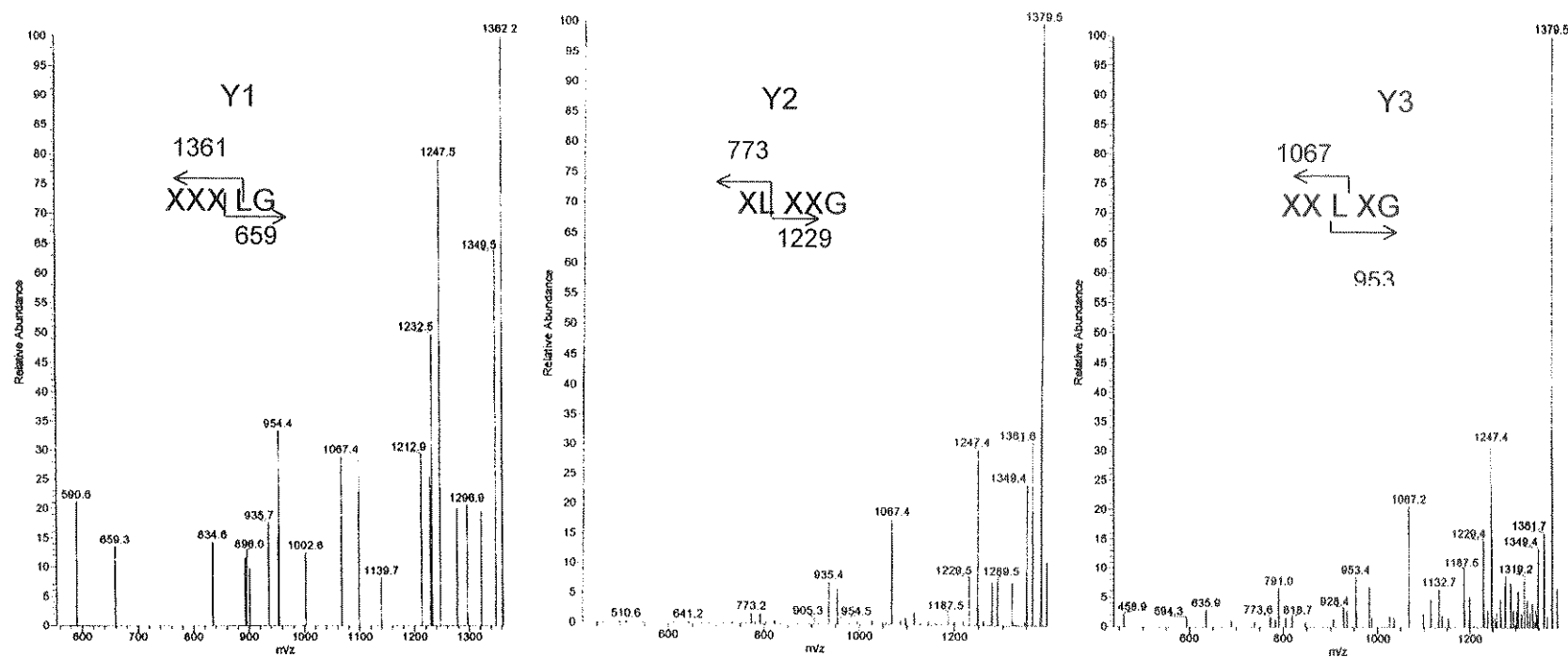


Figure 4. Electrospray mass spectrometry (ESI-MS) of the purified oligosaccharides from *H. courbaril* storage xyloglucan ($m/z=1541$). Some of the key ions for the characterisation of the oligosaccharide are identified.

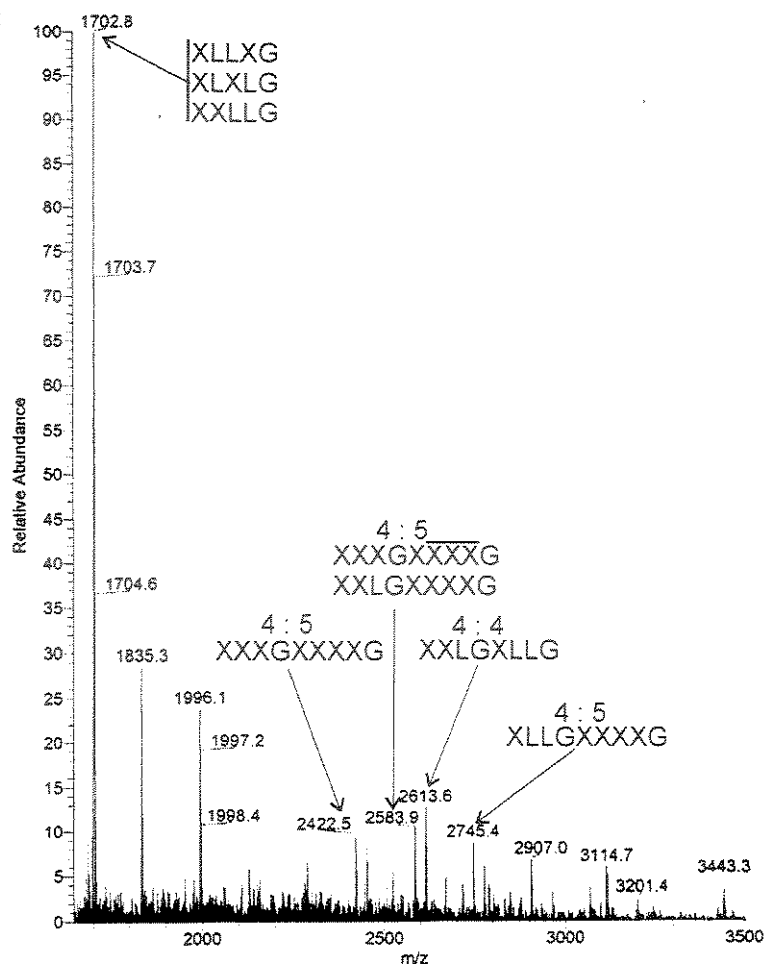


Figure 5. ESI-MS of the dimers of limit digest oligosaccharides of xyloglucan from *H. courbaril*. Some of the ions are indicated, as well as the number of glucoses in their main chain, showing the preferential alternation of four and five-glucose oligosaccharides. The line over XXXXG means that the galactosyl residue can be in any of the "X" indicated.

Artigo 2

Galactose branching modulates the action of cellulase on seed storage xyloglucans

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GALACTOSE BRANCHING MODULATES THE ACTION OF CELLULASE ON SEED STORAGE XYLOGLUCANS

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Running title: Action of cellulase on storage xyloglucans

abbreviations:

HPAEC-PAD, High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection.

Keywords: Xyloglucan, *Hymenaea courbaril* L., storage, polysaccharides, cell wall, endo β -glucanase.

Abstract:

Xyloglucan is a cell wall polysaccharide that has a main β -D-(1 $\rightarrow$ 4)-glucan backbone branched at regular intervals with α (1 $\rightarrow$ 6) linked D-xylopyranosyl or β -D-galactopyranosyl(1 $\rightarrow$ 2)D-xylopyranosyl residues. Despite the presence of other types of substitutions in the primary wall, these residues compromise almost all the structure of xyloglucan when it occurs as a storage polymer in seeds. Due to its cellulose-like backbone, this polymer is susceptible to the action of endo- β (1,4) glucanase (cellulase) at the non-branched glucosyl residues and the digestion of xyloglucan with cellulase and characterisation of the oligosaccharides generated have been widely used in the study of the structure of xyloglucan. Storage xyloglucans from *Copaifera langsdorffii* and *Hymenaea courbaril* were hydrolysed with cellulase and the equilibrium reached after 24 hours was analysed by Gel filtration and High Performance Anion Exchange Chromatography. The equilibrium contained limit digestion oligosaccharides (LDOs) and dimmers and trimmers of these LDOs that, despite their susceptibility to the enzyme, were not hydrolysed. Some of our data suggests that the side-chains of the polysaccharides can modulate the recognition of the fragments of xyloglucan by the enzyme: (1) the presence of a β -galactosidase in the system avoided the accumulation of these dimmers and trimmers, (2) less-branched LDOs are attached first by the enzyme, (3) polymers with different structure are hydrolysed at a different rate by the same enzyme. Considering that the branching pattern of the polysaccharide seems to have direct influence in the interaction of the enzyme with the substrate we suggest that, the structure of the polysaccharide would code at least part of the information required for its own metabolism.

Introduction

Seed xyloglucans have a main β -D-(1 $\rightarrow$ 4)-glucan backbone branched with α (1 $\rightarrow$ 6) linked D-xylopyranosyl or β -D-galactopyranosyl(1 $\rightarrow$ 2)D-xylopyranosyl residues. Except for the absence of terminal fucosyl units α -L-(1 $\rightarrow$ 2)-linked to the β -D-galactosyl groups, there is a remarkable similarity between seed reserve xyloglucan and structural xyloglucan from primary walls of dicotyledoneous vegetative tissues (Hayashi, 1989).

Using microbial cellulase, Kooiman (1961) found that tamarind seed xyloglucan is composed of the heptasaccharide Glc₄Xyl₃, with variation in the substitution with galactose residues. Following this finding, many authors have successfully used cellulase limit digest oligosaccharides (LDOs) as a diagnostic of the presence of xyloglucan in plant tissues. The experimental approach is to subject the soluble polysaccharide to the prolonged action of fungal endo- β -glucanases (cellulases) and perform analysis of the LDOs by TLC and/or HPAEC-PAD.

On the basis of the data produced until 1992, an unambiguous nomenclature for the structural blocks of xyloglucan has been proposed. This nomenclature takes the backbone as a central point. Unsubstituted glucose is assigned G; glucose residues branched with xylose are assigned X and if galactose is branching xylose, the trisaccharide is assigned L (Fry, York, Albersheim, Darvill, Hayashi, Joseleau, Kato, Lorences, MacLachlan, McNeil, Mort, Reid, Seitz, Selvendran, Voragen & White, 1993).

Usually, treatment of xyloglucan with different microbial cellulases (eg. *Trichoderma* spp or *Penicilium* spp) give similar results, (Vicken, Beldman, Nissen and Voragen, 1996). Another class of enzymes which is able to attack the main chain of xyloglucan is the XET-Xyloglucan Endo-Transglycosilases (Farkas, Sulova, Stratilova, Hanna & McLachlan, 1992). Fanutti, Gidley & Reid (1996) performed a comparative study using *Trichoderma* cellulase and nasturtium XET and showed that their pattern of action on xyloglucan is similar. Both produce a mixture of LDOs composed of XXXG, XLXG, XXLG and XXLG.

Vincken, York, Beldman and Voragen (1997a) reviewed the literature of usage of LDOs to characterise xyloglucan and suggested that these polymers can be classified into two groups, the Solanaceae type, which is based on XXGG blocks, and the others, which are based on XXXG blocks.

A comparative study of the fine structure of seed storage XGs (*Tamarindus indica*, *Tropaeolum majus* and *Copaifera langsdorffii*) has shown that they present a similar structural pattern of limit digest oligosaccharides, all being composed almost entirely of the Glc₄ subunits XXXG, XLXG, XXLXG and XLXLG (Buckeridge, Rocha, Reid & Dietrich, 1992). Buckeridge, Crombie, Mendes, Reid, Gidley and Vieira (1997) reported that the XG from seeds of *Hymenaea courbaril* displays unique structural features. Instead of being based on XXXG only, approximately 50% of the *H. courbaril* XG is composed of a family of oligosaccharides based on XXXXXG.

In the present work, we performed a study of the action of cellulase from *Trichoderma* on XXXXXG based xyloglucan from *Hymenaea courbaril* as compared with other storage XXXG type polymers. We observed that galactose modulates the action of *Trichoderma* cellulase on the storage xyloglucans from *Copaifera langsdorffii*, *Tamarindus indica* and *Hymenaea courbaril*. This modulation appears to be solely by structural constriction due to galactose distribution in the polymer.

Results

The digestion of *H. courbaril* storage xyloglucan with *Trichoderma* cellulase at low enzyme concentration rendered a mixture of oligosaccharides and low molecular mass polymers that appear as distinct peaks under chromatography on Biogel P-6 (Figure 1). This chromatographic pattern did not change after extended incubation up to 72 hours (data not shown) indicating that net hydrolysis stopped.

With the aim of checking the hypothesis that acetylation might have been a reason for resistance to further hydrolysis, *H. courbaril* XG was treated with NaOH. This hypothesis was ruled out on the basis of the fact that no change in the chromatographic pattern shown in Figure 1A was observed after treatment with 100mM

NaOH for 1 h prior to cellulase incubation (Figure 1B). This would have been expected if acetyl substitutions were present, since they are hydrolysed at high pH.

HPAEC analysis of the different P-6 fractions (FI, FIII and FIII) revealed that they are composed of a mixture of Glc₄Xyl₃ and Glc₅Xyl₄ LDOs (Figure 2). Furthermore, monosaccharide analyses of those fractions showed an enrichment in galactose in the higher molecular weight fractions (Table 1). Trace amounts of arabinose were found in the mixture of LDOs from *Hymenaea* but its occurrence did not reach the necessary frequency to explain the modulation of cellulase attack (see paper 3).

Both, increase in enzyme concentration or decrease in substrate concentration increased hydrolysis efficiency (Figure 3). However, the effect of enzyme concentration had a much greater effect since doubling the enzyme concentration led to disappearance of fragments larger than two LDOs (compare Figures 3A and B with 3C and D). The proportion of LDOs in the equilibrium reached after 24h with lower cellulase concentration did not reflect the proportion of oligosaccharides in the polymer. Fragments containing less “XL...” motives (galactose adjacent to the reducing end) appear to have been the last to be released from the polymer (compare the proportion of LDOs in figures 3A and D).

The fact that the reduction of substrate concentration led to an increase in hydrolysis raised the question whether the accumulation of lower molecular mass polymers might somehow be related to an inhibition effect. The oligosaccharides XXXG and XLLG were isolated from a mixture of LDOs from *T. indica* and *C. langsdorffii* respectively and their addition at an initial concentration of 0.5% (w/v) to the reaction mixture did not change the pattern of reaction products at all (results not shown). As the addition of FII or FIII did not interfere with the reaction either, our results ruled out the hypothesis of inhibition by the products.

The isolated fractions from Biogel P-6 were further incubated (24 and 48 h) with *Trichoderma* cellulase (Figure 4). Fractions FII and FIII were hydrolysed to produce the oligomers FI. These strongly suggest that FII and FIII are formed by multiples of FI oligosaccharides. We propose that FII are dimmers whereas FIII are trimmers of the XXXG/XXXXG families of oligosaccharides.

In order to test the hypothesis that galactose residues might be preventing further hydrolysis of the polymers, the storage xyloglucans from *H. courbaril* and *Tamarindus indica* were treated simultaneously with *Trichoderma* cellulase and nasturtium β -galactosidase (N β -gal) (Figure 5). When native xyloglucan polymers are treated with N β -gal alone, small amounts of galactose were released from the non-reducing terminals of the polymers (Alcântara et al. 1999), but no oligosaccharides could be detected (Figure 5). However, the prolonged action of a combination of *Trichoderma* cellulase and N β -gal with xyloglucan polymers from *H. courbaril* and *T. indica* completely hydrolysed FII to FI. At the same time as the β -1,4 linkages were hydrolysed, the oligosaccharides produced were consistently degalactosylated. This can be deduced from the presence of high proportions of XXXG and XXXXG in *H. courbaril* and XXXG in *T. indica* together with the production of high amounts of galactose from both LDOs (Figure 5). These experiments suggest that the presence of galactose at certain positions modulates cellulase action.

Figure 6 shows the patterns of hydrolysis of xyloglucans from *H. courbaril* and *C. langsdorffii* with *Trichoderma* cellulase at half of the usual concentration (25 mU). In these chromatograms, it can be clearly seen that even at early steps of hydrolysis (1h), fractions containing 2,3,4, 5 and higher blocks are produced. The reaction proceeded until it reaches an equilibrium and it is noticeable that speed of reaction strongly decreases as smaller fragments are produced.

Whereas in *H. courbaril* the equilibrium was reached after 3 h of reaction, for *C. langsdorffii* it was observed only after 9 h, indicating that *H. courbaril* xyloglucan is more labile than the one from *C. langsdorffii*.

Discussion

In this work we confirm and extend the hypothesis that galactose modulates cellulase activity on xyloglucans by structural constriction. Other authors have suggested this modulation (Vincken, Beldman and Voragen, 1977b; Fanutti, Gidley and

Reid, 1996) and with the use of storage xyloglucans, we obtained direct evidence for that by using a purified β -galactosidase from nasturtium cotyledons. Because *Hymenaea courbaril* XG was used as substrate, it was possible to evaluate the influence of galactose residues further ahead towards the reducing end of the chain, this due to the presence of XXXXG based polymer in *Hymenaea courbaril* xyloglucan (Buckeridge, Crombie, Mendes, Reid, Gidley and Vieira, 1997).

The dimmers and trimmers of LDOs (FII and FIII) were susceptible to the attack of cellulase. This indicates that the accumulation of these fragments is not due to their intrinsic resistance to hydrolysis and that the reaction reaches an equilibrium even when sites of hydrolysis are still available. The presence of β -galactosidase in the assay alters the equilibrium reached. When the galactoses are removed from the fragments of xyloglucan as they are produced, the hydrolysis proceed almost without accumulation of FII and FIII (Figure 5). This also points to the importance of the galactosyl side-chains in the modulation of the point of attack of the enzyme on the substrate. Apparently the accumulation of galactosylated oligosaccharides hinders the total hydrolysis of cellulase and the enzyme is not sensitive to the accumulation of free galactose. This highlights the importance of the interaction of the cellulase with the exo-hydrolases that occur in biological processes like fungal disassembling of the wall during pathogenesis and storage xyloglucan disassembling after seed germination. Although the exo-hydrolyses do not participate directly on the cellulase hydrolysis reaction, it seems to be capable to modulate the rate of reaction by altering the affinity of cellulase by the xyloglucan fragments. This supports the hypothesis that the activity of the exo-hydrolases might be the control points of the xyloglucan disassembling process (Crombie, Chengappa and Hellyer, 1998, Tiné, Cortelazzo and Buckeridge, 2000).

The proportion of LDOs obtained in the beginning of hydrolysis of *Copaifera langsdorffii* was very different from the proportion of LDOs in the total polymer (compare the proportion of XXXG and XLLG in Figures 3A and 3D). The mixture of LDOs was richer in XXXG in the beginning of the hydrolysis, although this oligosaccharide is not one of the major oligosaccharides in the total polysaccharide composition. This difference in proportion suggests that the attack of the enzyme on the

polymer is not random, and the enzyme hydrolyses first at the less branched regions (XXXG). This preferential attack of the enzyme upon certain oligosaccharides releases fragments that are progressively poorer in these preferential sites of hydrolysis.

Therefore, as hydrolysis proceed, the structure of the substrate changes continuously with the frequency of these high-affinity sites decreasing and the fragments produced become less susceptible to the enzyme attack. As a consequence, there appear to exist sites of action that, under low enzyme concentration, are attacked so slowly that are seen as being resistant to enzyme action towards the end of reaction.

The time course of hydrolysis of xyloglucans with different structures showed differences in the interaction between cellulase and xyloglucan. Under the conditions used, the xyloglucan of *H. courbaril* reaches the equilibrium in less than three hours, with little alteration in the average molecular weight of the fragments in the next 21 hours. The polysaccharide from *Copaifera langsdorffii*, on the other hand, reaches this point only between 3 and 9 hours. The storage xyloglucan from *H. courbaril* differ from the one of *C. langsdorffii* in at least two points: i) it has a series of five glucose-oligosaccharides and, ii) has less oligosaccharides with the “XL...” motif (see paper 1) in its structure. The presence of XXXXG-based oligosaccharides alters the distribution of the points susceptible to the attack of cellulase along the polymer. The fifth glucosyl residue in the structure of the oligosaccharide inverts the position of the next oligosaccharide and, therefore, the position of the next attack of the cellulase. This means that XXXG-based polymers are asymmetric (considering a flat conformation), with all the points susceptible to cellulase in the same side of the polymer. In the presence of XXXXG-based oligosaccharides, however, the inversion of the chain generates a more symmetric polymer, with points of hydrolysis on both sides of the chain. This could make the polymer of *H. courbaril* susceptible to the enzyme even if complexed with other polymers. The differences in amount of “XL...” motifs may also be an important element in the kinetics of hydrolysis of the polymer, since the presence of this galactose adjacent to the non-reducing end seems to play an important role in the interaction of the enzyme with the substrate.

Fanuti, Gidley & Reid (1996) performed a study of the subsite recognition of nasturtium XET. They found that the positions of the galactose residues on each LDO appear to modulate differentially the attack of the enzyme. Vincken, Beldman and Voragen (1997b) studied the substrate specificity of endo-glucanases with a focus on the xyloglucanase activity. They found that galactose and fucose interfere with the action of the enzymes, usually by decreasing reaction speed. Together with our results, these data indicate that the interaction of the endo-glucanases (either from plants or from fungi) and xyloglucan is dependant on the branching pattern, which is a feature of the polysaccharide. This means that the polymer is capable to modulate the action of these enzymes upon the substrate and at least part of the information required for xyloglucan degradation is “recorded” in the polymer itself, i.e. on the branching pattern of the polymer produced during its biosynthesis.

Our results suggest that the fine structure of xyloglucans, especially regarding galactose positioning, seem to determine the speed with which cellulase attacks different points at the molecule. Although in the present work we did not perform experiments with XETs, Fanuti, Gidley and Reid. (1991) found similar effects of galactose on cellulase and XET hydrolyses of tamarind xyloglucan. In the present work, we used *Copaifera*'s xyloglucan instead, but recent results from our laboratory show that the fine structure of these two xyloglucans (the proportion of oligosaccharides) is almost identical (C.O.Silva, D.U. Lima & M.S. Buckeridge, unpublished).

The results presented above have interesting implications for the biological roles of storage xyloglucans. First, for a seed storage system where the proportion cellulose/hemicellulose is very altered in relation to the primary wall, xyloglucan molecules have to maintain self interactivity and galactose is thought to hinder it (Levy, 1991). On the other hand, positioning of the galactosyl residues are important to give higher solubility to the molecules, i.e. as higher the galactose content, the higher the water solubility (Buckeridge, Santos and Tiné, 2000) so that a polymer with very high galactose proportions would be promptly soluble but according to our results would also be hydrolysed very slowly. The second implication regards the attack of a fungi to xyloglucan containing seeds. The existence of branching points that modulate

degradation by cellulase, such as the one studied in the present work, poses the problem of increase of complexity to the micro-organism evolutionary performance and denotes important aspects of the co-evolution of legumes, that store xyloglucan, and fungi capable to attack such seeds.

Materials and Methods

Plant material and polysaccharide extraction

Seeds of *Hymenaea courbaril* L. were provided by the Seed Department of the Institute of Botany at São Paulo (Brazil) and those of *Copaifera langsdorffii* were harvested at the São Paulo Botanical Garden. The polysaccharides were extracted from cotyledon powders with water (1% w/v) at 80°C for 8 h with constant stirring. After filtration, 3 volumes of ethanol were added to the aqueous extracts, kept overnight at 5°C and centrifuged (12,000 g for 15 min at 5°C). The pellet was washed with acetone, dried at room temperature and, after resuspension in water, freeze-dried.

Treatment of xyloglucan of *Hymenaea courbaril* with NaOH

The water soluble xyloglucan from *H. courbaril* was incubated with 100 mM NaOH supplemented with 0.01% NaBH₄ for 30 min at room temperature. The solution was neutralised with acetic acid and deionised with cationic and anionic Dowex resins. After freeze drying, the polymer was applied onto a Biogel P-6 column as described below.

Analysis by gel filtration

Five mL of a 0.5% (w/v) solution of xyloglucan in Na⁺ Acetate buffer (50mM pH 5.0) were digested with 200µl of a 1:100 dilution of *Trichoderma viride* cellulase (Megazyme, 500 U mL⁻¹ in 3.2 M ammonium sulfate, pH 7.0) for 24 h at 30°C. The solution of oligosaccharides produced was loaded onto a Biogel P-6 (BioRad) column

(1.5 x 170 cm) and eluted with a 0.05% solution of NaN_3 . Fractions of 1 mL were collected and carbohydrates were quantitatively analysed by phenol-sulfuric method (Dubois, Gilles, Hamilton, Rebers and Smith, 1956).

Monosaccharide analysis

Pooled fractions had their total sugar determined as above and a volume corresponding to 1 mg was freeze-dried and hydrolysed to their monosaccharide constituents using 72% H_2SO_4 -4% H_2SO_4 (Saeman, Buhl and Harris, 1945). The monosaccharide solution was neutralised with 60 μl of a 50% (w/w) solution of NaOH (Fischer Scientific) to a pH between 6 and 8 and the monosaccharides were analysed on a CarboPak PA1 column (Dionex DX-500 system) using water as eluent ($1\text{ml}\cdot\text{min}^{-1}$) for 50 min with a post-column with 500mM NaOH ($1\text{ml}\cdot\text{min}^{-1}$).

Analysis of xyloglucan oligosaccharides

Xyloglucan oligosaccharides were analysed by HPAEC-PAD on a Dionex system DX-500 using a CarboPak PA100 column and detected by pulsed amperometric detection (PAD). The samples were eluted with a gradient of sodium acetate (from 35 to 75 mM) in sodium hydroxide (88mM) and with a flow of 0.9 mL/min. High molecular weight fragments were analysed on the same system with an acetate gradient from 50 to 330 mM of Na Acetate in NaOH 88mM for 50 min ($0.9\text{ml}\cdot\text{min}^{-1}$).

Digestion with Cellulase and β -galactosidase

For the production of high molecular weight fragments of xyloglucan, solutions of storage xyloglucans (0.1, 0.5 or 1.0 %, w/v) were prepared on 50mM Na acetate buffer pH 5.0. Two hundred microliters of the polysaccharide solution were incubated with 50 μl of cellulase ($1.0\text{U}\cdot\text{ml}^{-1}$). For assays with lower concentration of cellulase, the solution of cellulase used was $0.5\text{U}\cdot\text{ml}^{-1}$. For incubations with β -galactosidase, the same incubation system was used, with the addition of 20 μl of *Tropaeolum majus* β -galactosidase purified according to Edwards, Bowman, Dea and Reid (1998).

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Table 1. Monosaccharide composition of the pooled fractions from Bio-Gel P-6 (cellulase limit digest oligosaccharides) from storage xyloglucan of *Hymenaea courbaril*.

Fractions	Glucose	Xylose	Galactose
FI	5,0	3,0	1,0
FII	5,0	3,1	1,6
FIII	5,0	2,9	2,9

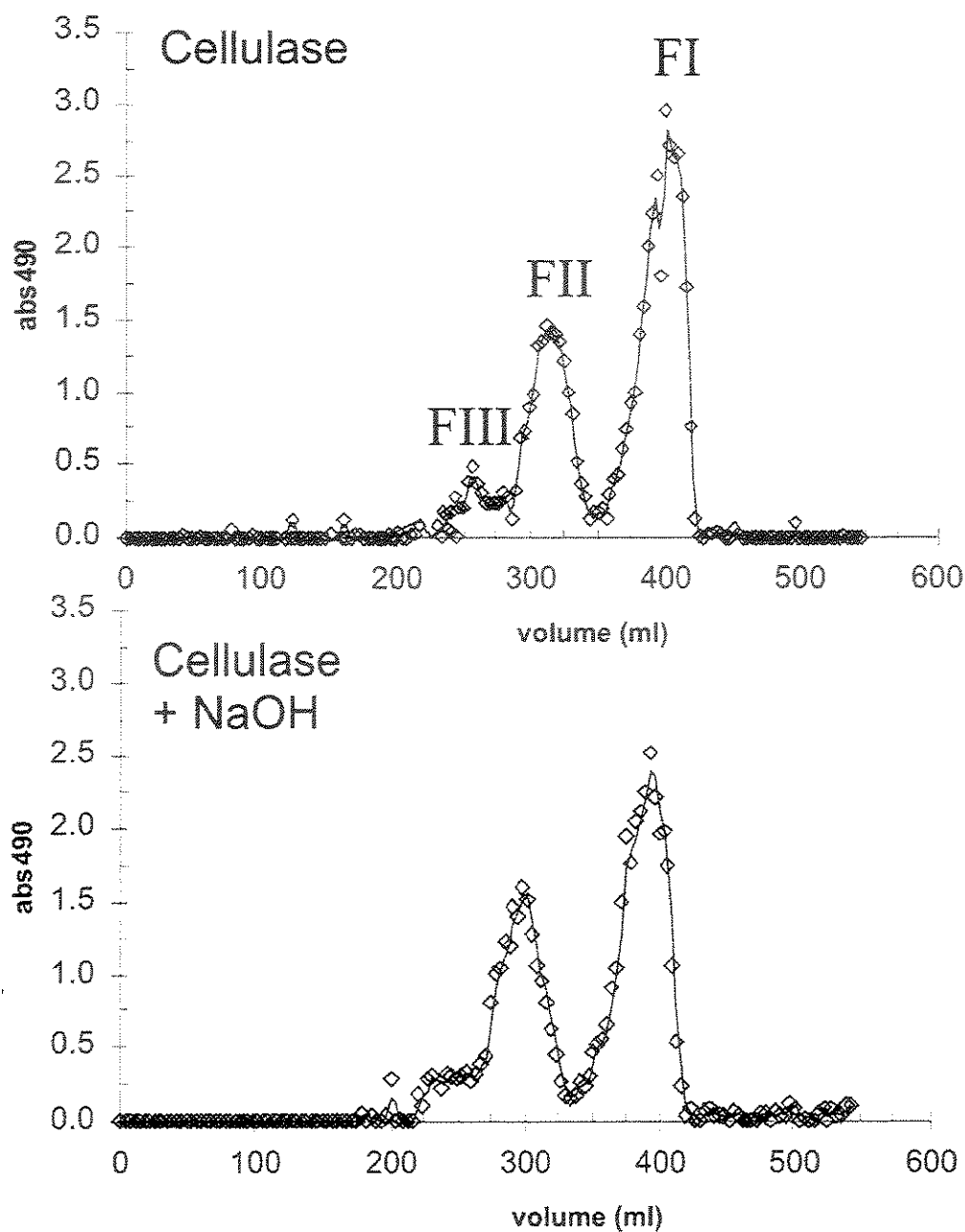


Figure 1. Gel chromatography on Biogel P-6 of exhaustive digestion with cellulase from *Trichoderma* sp (Megazyme, Australia) under suboptimal enzyme concentration. A – pattern of oligosaccharides obtained with the native polysaccharide; B – polysaccharide was treated with 100mM NaOH for 1h prior to dialysis and enzyme assay.

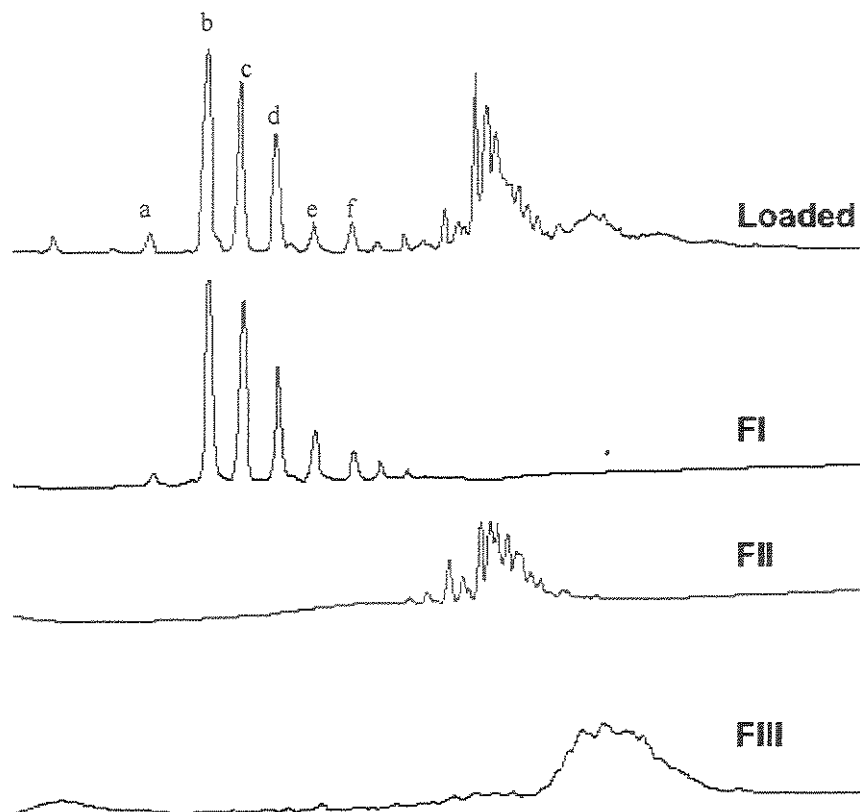


Figure 2. Analysis by High Performance Anion Exchange Chromatography of the three fractions isolated by Biogel P-6 (FI, FII and FIII). The top chromatogram corresponds to the mixture of oligosaccharides loaded into the column. The peaks in the limit digest oligosaccharides fraction are: a=XXXG, b=XXLG, c=XLLG+XXXXG, d=XXXLG, e=XLXXG, f=XXLXG. Peaks d, e and f were identified according to paper 1)

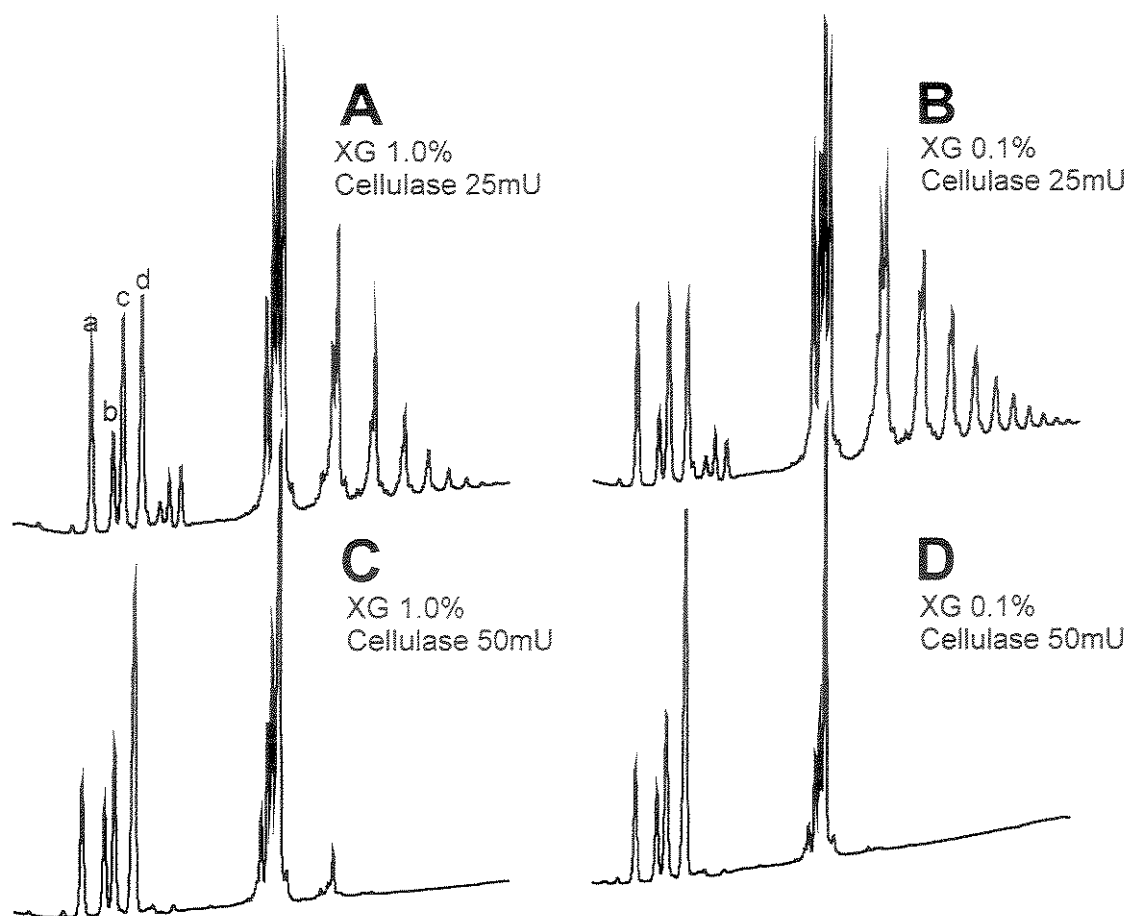


Figure 3. Effect of enzyme and substrate concentration on the rate of hydrolysis of xyloglucan. Two concentrations of *Copaifera langsdorffii* xyloglucan (1.0 and 0.1%) were hydrolysed with 50 and 25 miliunits (mU) of cellulase. The oligosaccharides in the LDO mixture are: a=XXXG, b=XLXG, c=XXLG and d=XLLG.

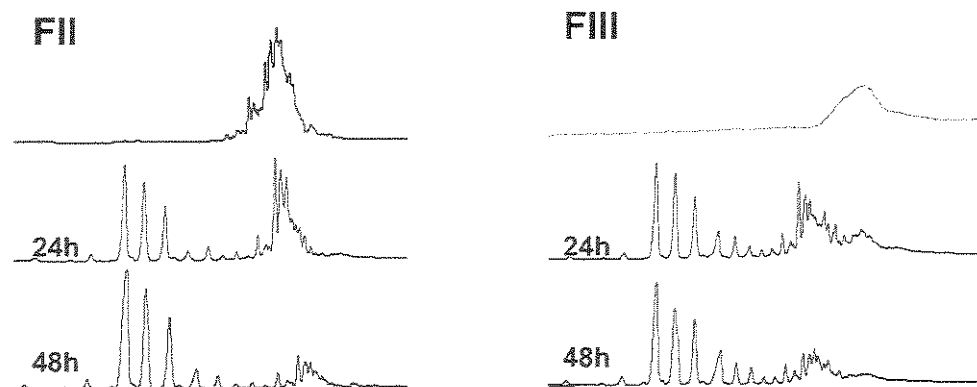


Figure 4. Hydrolysis of the fragments of storage xyloglucan from *Hymenaea courbaril* (fractions FII and FIII from Biogel P-6) with *Trichoderma* cellulase. Both fragments are susceptible to hydrolysis with the enzyme, producing the FI oligosaccharides.

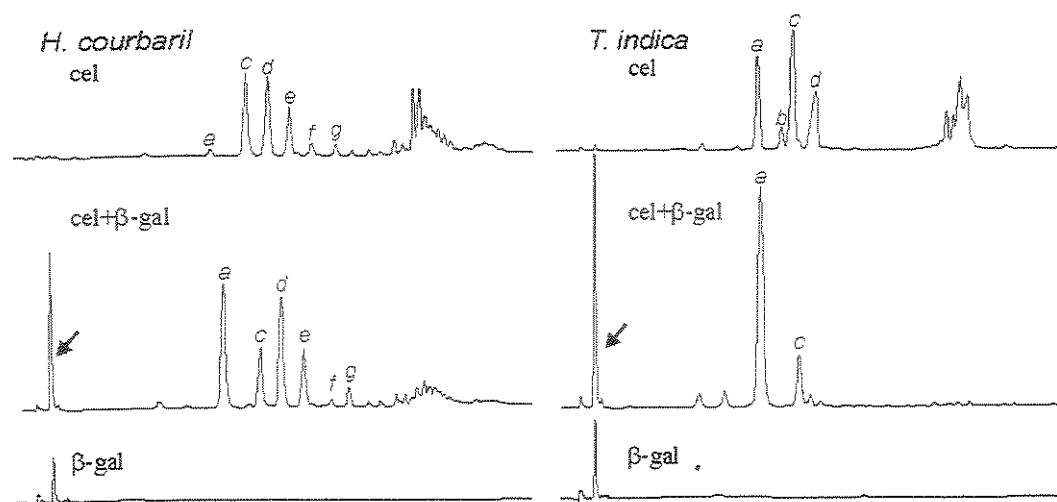


Figure 5. Hydrolysis of *H. courbaril* and *T. indica* storage xyloglucans with cellulase, cellulase plus nasturtium β -galactosidase and nasturtium β -galactosidase only for 24 h. The arrow points to the free galactose released, $a=XXXG$, $b=XLXG$, $c=XXLG$, $d=XLLG+XXXXG$ (in *T. indica*, this peak contains only $XLLG$), $e=XXXLG$, $f=XLXXG$, $g=XXLXG$.

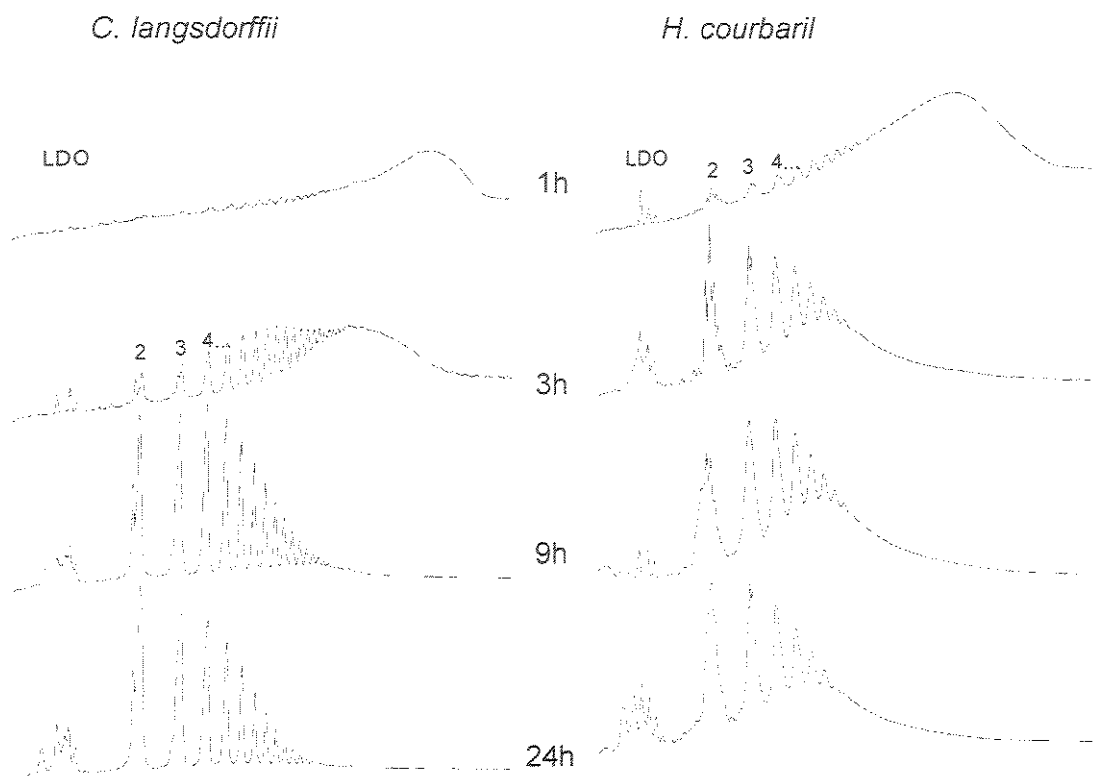


Figure 6. Time course of hydrolysis of storage xyloglucans from *H. courbaril* and *Copaifera langsdorffii* from 1 to 24 h. The numbers above the peaks indicates the number of oligosaccharides in the fragment. LDO= cellulase limit digest oligosaccharides.

Artigo 3

Detection and Subcellular Localisation of Fucosylated and Non-Fucosylated Seed Xyloglucan in Storage Cell Walls of Leguminous Seeds

A ser submetido à revista “Plant Physiology”.

Detection and Subcellular Localisation of Fucosylated and Non-Fucosylated Seed
Xyloglucan in Storage Cell Walls of Leguminous Seeds

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Abbreviations: KBPS 10 mM potassium phosphate buffer, pH 7.2, containing 500 mM NaCl; XG, xyloglucan; TEM, Transmission Electron Microscopy.

Running title: Detection and localisation of fucosylated xyloglucan in storage walls

Abstract: The plant cell wall is a composite of polysaccharides, proteins and phenolic compounds, which vary according to the species, tissue, cell and specific wall within a single cell. Among the hemicellulosic polymers, XG is of universal occurrence in dicots. It is composed of a cellulosic backbone of β -1,4-glucan carrying branches of α -1,6 xylosyl residues to which are attached galactosyl residues linked β -1,2. Terminal fucosyl residues may also be present at the ends of the galactose-containing branches. Fucose-containing side-chains are not typically found in seed storage XG. When treated with fungal cellulase, XGs are cleaved into oligomers that can be used as a fingerprint of the polymer. After separation of oligomers from cotyledons of *Hymenaea courbaril* on Biogel P-6, they were subjected to monosaccharide analysis by HPAEC-PAD. Small amounts of fucose were detected. These results indicate that, besides the unfucosylated seed storage XG, small amounts of primary cell wall fucosylated XG is present. Sections of cotyledons of *Hymenaea courbaril* were labelled with the CCRC-M1 monoclonal antibody that specifically recognises the fucosyl- α -(1,2)-galactosyl motifs in primary cell wall XG. Two labelled wall layers could be observed by light microscopy: one inner layer of primary cell wall near the plasma membrane and one outer layer near the middle lamella. This indicates the presence of at least two populations of xyloglucan in different layers in the storage wall: a structural fucosylated xyloglucan and a storage unfucosylated xyloglucan. The two distinct populations were also detected in other xyloglucan storing cells in seeds of *Copaifera langsdorffii*, *Tamarindus indica* and *Tropaeolum majus*. These results were confirmed by immunogold staining, which showed clear boundaries between the outer and inner primary walls and the storage wall, suggesting a rapid shift in the biosynthetic mechanism upon change from the synthesis of the structural and the storage polymers. During storage mobilisation, only the storage XG disappears whereas the two primary walls collapse. These results highlight the rather complex topology of XGs with different fine structures within the wall and suggest that storage mobilisation must be finely controlled to selectively mobilise only the appropriate polysaccharide within storage tissues.

Introduction

Many legume seeds contain carbohydrates as storage compounds that are used by the embryo/plantlet during and after germination. Usually, the first reserves to be broken down are the raffinose family oligosaccharides, whereas cell wall polysaccharides such as galactomannan (Reid, 1985), galactan (Crawshaw and Reid, 1984) and XG (Reis et al., 1987; Buckeridge et al., 1992) are thought to be reserves for the growing plantlet, being degraded after germination (Buckeridge and Reid, 1996). Among legumes, some members of the subfamily Caesalpinoideae are known to accumulate XG in the cotyledons (Kooiman, 1961; Polhill and Raven, 1981; Buckeridge and Dietrich, 1990; Buckeridge et al., 1995). These polymers are present in storage cell walls of species such as *Tamarindus indica* (tamarind) (Koiman, 1961), *Copaifera langsdorffii* and *Hymenaea courbaril* (Buckeridge and Dietrich, 1990; Buckeridge et al., 1992). In these walls, the proportion of hemicellulose and other polymers is highly deviated from the proportions of a growing wall (Buckeridge et al., 2000). Although the existence of these storage walls has been described a long time ago (Vogel and Schleiden, 1839), the question of how a solid wall is assembled with little or no cellulose remains to be elucidated.

Seed storage XG is composed of a cellulosic β -(1,4)-glucan backbone, carrying branches of α -(1,6)-xylopyranosyl (forming with the glucose of the backbone a disaccharide assigned X) or β -(1,2)-D-galactopyranosyl- α -(1,6)-D-xylopyranosyl (forming a trisaccharide assigned L) (Fry et al., 1993). Except for the absence of terminal fucosyl units α -(1,2)-linked to the β -D-galactopyranosyl groups, there is a remarkable similarity between seed storage and structural XGs from primary walls of dicots (Hayashi et al., 1989). A comparative study of the fine structure of seed storage xyloglucans (*Tropaeolum majus*, *Tamarindus indica* and *Copaifera langsdorffii*) has shown that they present similar structural patterns of limit digest oligosaccharides, all being composed almost entirely of the Glc₄ subunits XXXG, XLXG, XXLG and XLLG (Buckeridge et al., 1992). The only exception was the XG from seeds of the tropical tree *Hymenaea courbaril*, which displays unique structural features. Instead of being based

on XXXG only, approximately 50% of the *H. courbaril* XG are composed of a family of oligosaccharides based on XXXXG (Buckeridge et al., 1997).

Recently, Tiné et al. (2000a) described the events that take place during XG mobilisation in cotyledons of developing plantlets of *H. courbaril*. They found that the hydrolytic activity of XET is highly dependent on the presence of XG oligosaccharides and proposed that β -galactosidase could be one of the control points of the disassembling process. The authors demonstrated that a small portion of the XG of seeds of *H. courbaril* that can only be extracted with alkali has a different monosaccharide composition and does not decline after germination. The increase observed in the proportion of arabinose in the storage XG after mobilisation was suggested to be due to the decline in the storage polymer content and to a proportional increase in the contamination with a “structural” arabinose-containing polymer. These data suggested that in the seeds of this species at least two types of XG are present in the storage wall: (i) a storage XG, highly soluble and poor in arabinose and (ii) a structural XG, less soluble and richer in arabinose, as found in Tiné et al. (2000).

In fruits of *Tropaeolum majus*, Desveaux et al. (1998) described the presence of large deposits of non-fucosylated XG in the periplasmic spaces of cotyledon cells and small amounts fucosylated XG in the structural fraction that is bound to cellulose in the primary walls. The storage XG is synthesised without fucose, although it can be fucosylated by a nasturtium transferase *in vitro*, and it is deposited as a layer underneath the “structural layer”.

In *H. courbaril*, when the storage XG was treated with fungal cellulase and cleaved into oligomers, small amounts of fucose were detected. These results indicate that, besides the unfucosylated seed storage XG, small amounts of primary cell wall fucosylated XG are present. This work demonstrates the presence of fucose in the storage cell walls of seeds of *Hymenaea courbaril* L. and localises this residue at subcellular level using the monoclonal antibody CCRC-M1. Our data show that not only two classes of XG occur in the storage wall but also that they are chemically and topologically distinct, with the “storage” polymer trapped between two layers of

“structural” XG. Comparison with two other legumes *Copaifera langsdorffii*, *Tamarindus indica* and with the non-legume *Tropaeolum majus* is also included.

Results

Composition of XG Oligosaccharides of *H. courbaril* and *C. langsdorffii*

The extraction of the storage polysaccharides from *H. courbaril* and *C. langsdorffii* with hot water followed by hydrolysis with *Trichoderma* cellulase generated gel-filtration patterns typical of the storage XGs (Fig. 1). Further hydrolysis of Biogel P-6 isolated fractions with cellulase generated the typical structural blocks of XGs, i.e. the XXXG family based structural blocks for *C. langsdorffii* and the mixture of XXXG and XXXXG families of oligosaccharides for *H. courbaril* (not shown). The structure of the polysaccharide was confirmed by GC/MS analysis (Table I) and no unusual glycosyl linkages for a storage xyloglucan were detected.

The fractions obtained after Biogel P-6 were isolated and had their monosaccharide composition analysed by hydrolysis and HPAEC-PAD (Table II). In all fractions, besides glucose, xylose and galactose, small proportions of arabinose and fucose were detected. Since we have used purified *Trichoderma* cellulase and fucose and arabinose were found to be present in the limit digest oligosaccharides, it is very likely that they came from XG. The possibility that fucose might have come from rhamnogalacturonan I may be discarded since incubation of this polymer with purified cellulase would hardly release fragments in the range of 3000 Da and residues typical of RGI were not detected in the GC/MS analysis.

Distribution of Fucose Epitopes in the Cotyledons Cell Walls

Using a fucose recognising antibody (CCRC-M1) (Puhlmann et al., 1994), fucose has been detected in the storage cell walls of seeds of *C. langsdorffii*, *Tamarindus indica*, *Tropaeolum majus* (Fig. 2), and *H. courbaril* (Fig. 3). In the quiescent seeds of *C. langsdorffii* there is a thin outer layer in the storage wall that is recognised by the CCRC-M1 antibody (and, therefore, contains fucose) and a thick layer where no

labelling can be visualised (Fig. 2A). In *H. courbaril* a second layer of fucosylated wall can also be observed adjacent to the plasma membrane (Fig. 3). *Tamarindus indica* and *Tropaeolum majus* (Figs. 2B and 2C, respectively), used for comparison, showed the same pattern observed for *H. courbaril*, where the unfucosylated XG is deposited between two layers of fucosylated XG.

The CCRC-M1 antibody used could, in principle, recognise the fucosyl side-chains of rhamnogalacturonan I. However, the distribution of the labelling along the outer layer did not match with the acidic polysaccharide distribution observed for this species: mainly in the pit-fields and contacts between two cells (Tin   et al., 2000b).

Changes in storage and structural layers during xyloglucan mobilisation

Figure 3 shows clearly that in *H. courbaril*, both layers of fucosylated XGs are observed and only the non-labelled middle layer is mobilised after germination. This indicates that the “structural” and the “storage” XGs are different not only in chemical composition (presence of fucose), but also in subcellular localisation. As shown in Figure 4, the fucosylated inner layer is not disassembled by the hydrolytic machinery involved in storage mobilisation and maintains its integrity even after the complete degradation of the storage XG.

The sharp transition from one layer to the other (Fig. 4) indicates that the two populations of XG are clearly defined and the shift in the biosynthesis from one to the other must be very well defined as well. The gold particles labelled the inner and outer walls, with no labelling in the storage wall. The electron densities of the storage and structural layers are also different, suggesting that other differences may exist, such as the density of cellulose in the structural and storage layers.

Figures 4D and E show the presence of degradation pockets in the storage layer. At this stage, the pockets do not have a preferential location close to plasma membrane but the mobilisation of the storage XG seems to occur throughout the entire layer.

Discussion

Fucosyl residues were detected in the oligosaccharides of storage xyloglucan from seeds of *C. langsdorffii* and *H. courbaril*. The immunolocalisation of the residue in the wall of these seeds showed that the fucosylated and the unfucosylated xyloglucans are not evenly deposited in the wall, but appear as distinct layers. Thus, the presence of fucose in XG, even in very small amounts, might be related either to minor activity of α -fucosyl transferase during storage XG biosynthesis or to a small contamination of the extracted XG with some structural XG from the primary wall. In cotyledons of *T. majus*, *T. indica* and *H. courbaril*, an inner layer of fucosylated xyloglucan close to the plasma membrane was also observed. This suggests that the presence of this layer is not related to the occurrence of the unusual structure (5-glucose oligosaccharides) of the *H. courbaril* storage xyloglucan., previously reported (Buckeridge et al., 1997), but a rather general feature of xyloglucan-containing storage walls.

The previous evidences of the presence of multiple layers in storage walls of *Tamarindus indica* and *Tropaeolum majus* were indirectly obtained by Reis et al. (1985) and Desveaux et al. (1988) based on electron density of the layers and chemical analysis of the water-extracted polysaccharides. The results shown in Figures 2 and 3 are the first direct evidence of differences in composition among these layers by *in situ* detection: the XG from the inner and the outer layers have fucosyl substitutions whereas the middle layer is characterised by the absence of these sugar residues. Desveaux et al. (1998) described a swelling of the outer layer in *Tropaeolum majus* during the deposition of the storage layer. This dispersion of the microfibrils induced by the thickening of the wall may be the reason for the existence of an inner layer that maintains the integrity of the wall throughout the whole process of storage layer deposition and mobilisation. In *C. langsdorffii*, however, an inner layer labelled with CCRC-M1 was not observed. Considering that in these seeds the storage XG is mobilised by a mechanism similar to the other species (Alcântara et al., 1999), the presence of this inner layer seem not to be essential to the physiological role of the storage wall, and at the same time requires

further investigation to understand which evolutionary forces might have been involved in the selection of such a character.

The presence of these layers characterise the existence of at least two populations of xyloglucan in the wall. These populations are distinct in function, subcellular localisation and composition. The storage xyloglucan is very soluble, lack fucose and arabinose (as suggested by Tiné et al., 2000a), whereas the structural xyloglucan is less soluble (despite the small contamination in the water soluble), with fucosyl and arabinosyl side chains. The assembling of a functional storage wall with xyloglucan in seeds, therefore, possibly requires a complex modulation of the fucosyl transferase during XG biosynthesis.

This chemical distinction of the xyloglucan is important to separate the functions of the two polymers. In *H. courbaril*, the proposed control points of the disassembling process seem to be related to the exo-hydrolases (Tiné et al., 2000a). These enzymes act sequentially on the fragments of xyloglucan produced by XET. The β -galactosidase, β -glucosidase and α -xylosidase alternate themselves in cycles of disassembling, but each enzyme can only hydrolyse its corresponding substrate residue (galactosyl, xylosyl or glucosyl) after the action of the previous enzyme (Crombie, 1998). It may be suggested that the presence of fucosyl and arabinosyl in the structural xyloglucan may hinder the attack of the exo-hydrolases present during the mobilisation of the storage xyloglucan. This could be an explanation for the absence of fucosyl from the storage xyloglucans, as well as the avoidance of biological effects of the fucosylated oligosaccharides. In other words, the differences in the monosaccharide composition, therefore, can be a simple mechanism to separate the metabolism of the two populations of xyloglucan.

During seed development, the parenchyma cells go through at least two different metabolic states: the “growth and division” and the “storage” state. During morphogenesis, the tissues are growing and differentiating. When the seed is close to its final size, the metabolic state of the cells change and they turn their metabolic pathways to the accumulation of storage compounds. As a consequence, the dry mass of the seed increases with little change in the final size. This shift in the metabolic state is followed

by changes in the ratio sucrose/monosaccharides (Weber et al., 1997). Clearly, this shift also involves changes in the cell wall biosynthesis with changes in the fucosyl-transferase activity. The structural polysaccharides are important to maintain the integrity of the tissues during growth, but as soon as cell extension stops, the storage polysaccharide (unfucosylated) is deposited. It is not clear whether the storage layer is deposited below the structural layer and a second structural layer is deposited later on to maintain the mechanical properties of the wall or whether the storage layer is deposited between two structural layers previously assembled (in the case of the species that have an inner wall). In both alternatives, a complex synchronisation of the biosynthetic apparatus with and the previously deposited layers and the cell metabolism must occur.

Material and Methods

Plant material

Seeds of *Hymenaea courbaril* L. were provided by the Seed Department of the Institute of Botany at São Paulo (Brazil) and those of *Copaifera langsdorffii* were harvested at the São Paulo Botanical Garden. *Tamarindus indica* seeds were purchased at local market and seeds of *Tropaeolum majus* were purchased from Dæhnfeldt, Denmark, and both were used only for comparison in the fluorescent immunolabeling analysis.

XG Extraction

XG was prepared from the powder of seeds of *H. courbaril* and *C. langsdorffii* as described by Buckeridge et al. (1997). Five mL of a 0.5% (w/v) solution of XG in Na⁺ Acetate buffer (50mM pH 5.0) were digested with 200µl of a 1:100 dilution of *Trichoderma viride* cellulase (Megazyme, 500 U mL⁻¹ in 3.2 M ammonium sulfate, pH 7.0) for 24 h at 30°C. The solution of oligosaccharides produced was loaded onto a Biogel P-6 (BioRad) column (1.5 x 170 cm) and eluted with a 0.05% solution of NaN₃.

Fractions of 1 mL were collected and carbohydrates were quantitatively analysed by phenol-sulfuric method (Dubois et al., 1956).

Sugar Composition Analysis

Volumes corresponding to 1 mg of pooled fractions (I, II, III) were freeze-dried in 15 mL tubes and hydrolysed with sulfuric acid according to Saeman et al. (1945). The monosaccharides produced were analysed by HPAEC-PAD in a Carbowpak PA-1 column (Dionex) with 20 mM NaOH for 40 min (0.8 mL min^{-1}).

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 (1993). Partly methylated alditol acetates were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry as described by Carpita and Shea (1989).

Growth Conditions of *H. courbaril*

Seeds of *H. courbaril* were scarified individually by abrading the seed coat with sand paper, weighed, soaked in commercial sodium hypochlorite for 5 min, washed with tap water for 10 min, soaked in distilled water for 12 h and imbibed in a tray covered with moist filter paper. After germination, the seeds were transferred to pots filled with sand:vermiculite (2:1). Trays with the seeds regularly spaced were kept at 25°C under a 12 h photoperiod. The cotyledons were collected 14, 35 and 48 days after planting (before, during and after XG mobilisation, respectively) and used for immunolabeling procedures. Quiescent seeds of *H. courbaril*, *C. langsdorfii*, *Tropaeum majus*, and *Tamarindus indica* were also processed for comparison.

Fixation and Embedding

Cotyledon pieces of *H. courbaril* measuring approximately 2 mm length at all sides were fixed with *p*-formaldehyde (2.5%) /glutaraldehyde (0.5 %) in phosphate buffer 0.05M pH 7.2 for 4 h at room temperature under vacuum, washed 3 times in the same buffer, post-fixed for 1 h in the same buffer containing 1% (w/v) osmium

tetroxide. The fixed tissue was again rinsed 3 times in phosphate buffer before dehydration in a series of ethanol (20, 35, 50, 60, 70, 80, 90, 100% (v/v) for 30 min at each step) at room temperature. The dehydrated tissue was gradually infiltrated with LR White embedding resin (Ted Pella Inc., Redding, CA) according to the following schedule: 20, 40, 60 and 80% (v/v) LR White: ethanol, each step for 24 h with a change of resin every 8 h and 100% LR White for 24 h with a change of resin every 8 h. The infiltrated tissue was transferred to BEEM capsules (Ted Pella) containing 100% LR White for embedding. Polymerisation was accomplished by heating at 60°C for 48 h. For the other three species only quiescent seeds were used. They were processed as described above except that fixation was performed in 50mM cacodilate buffer with 150 mM glycine overnight.

Immunofluorescence Procedures

All incubations were carried out at room temperature unless otherwise indicated. For *H. courbaril*, transversal thick sections (250 nm) were cut with an MT 6000-XL (RMC Inc., Tucson, AZ) ultramicrotome and mounted on glass microscope slides previously coated with a solution of 0.4%(w/v) gelatin, 0.04% (w/v) Chrom-alum (Fisher), and 0.02% (w/v) Sodium Azide. For the other three species, thicker sections (1500 nm) were used. The sections were hydrated by placing droplets of KBPS on them for 10 min. Non-specific antibody-binding sites on the sections were blocked by incubation with 3% (w/v) nonfat dried milk in KBPS for 45 min. The sections were then incubated for 60 min on droplets of CCRC-M1 antibody diluted 1:1 (v/v) in KBPS, followed by a 30 s rinse in KBPS 3 times. The sections were then incubated in droplets of goat anti-mouse IgG-fluorescein isothiocyanate conjugate for 1h. The sections were washed with one 30 s rinse each of KBPS and distilled water. Epifluorescence of the immunostained tissue sections was viewed through a Zeiss UV filter set (excitation filter 450-490nm, beam splitter 510nm, barrier filter 520 nm) and photographed with T-max 100 35-mm (Kodak).

Some control experiments were performed by omission of the primary antibody. For the competition assays, a 2M fucose in KBPS solution was incubated together with the primary antibody.

EM Procedures

Thin sections (<100nm) were mounted on gilded copper slot grids and labelled as described for immunofluorescence microscopy, except that the secondary antibody was gold anti-mouse IgG conjugated to 15-nm colloidal gold (prepared as described by Freshour et al., 1996). After immunolabeling, the sections were post-stained for 3 min with 4% (w/v) aqueous uranyl acetate and for 30 s with lead citrate. The sections were examined at 80 kV with a Zeiss EM 902A electron microscope.

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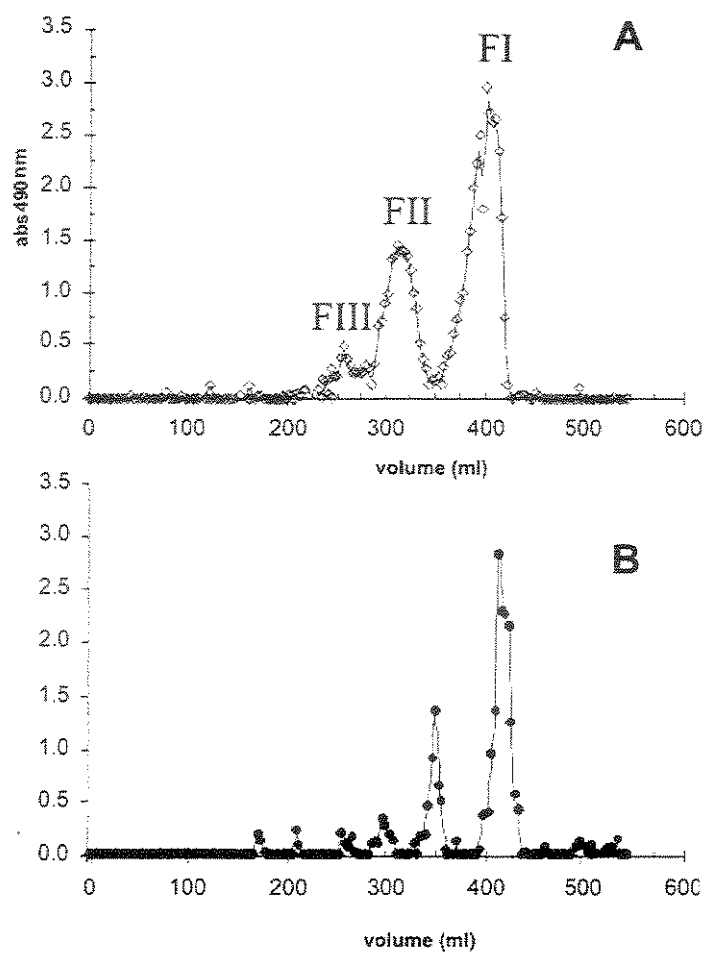


Figure 1. Biogel P-6 profile of the storage XG from *H. courbaril* (A) and *C. langsdorffi* (B) after hydrolysis with fungal cellulase. The peaks I, II and III refer to the number of limit digest oligosaccharides present in the fragment.

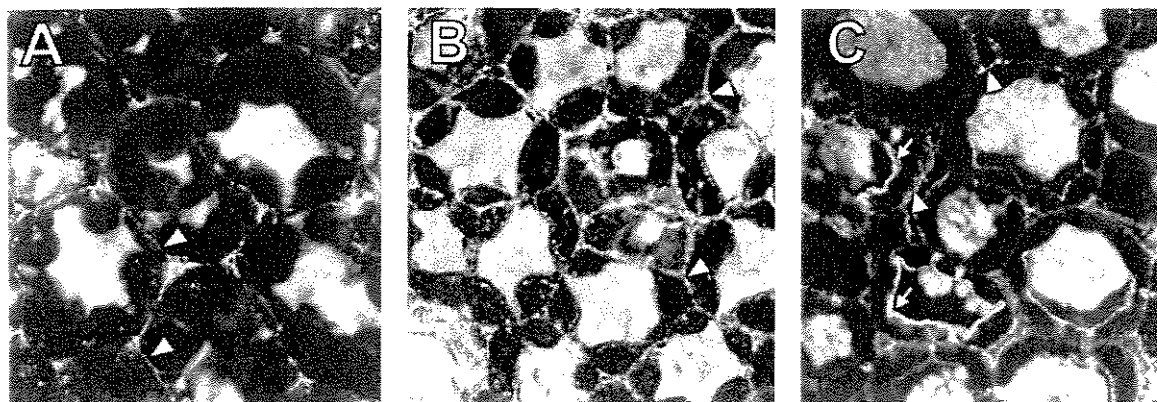


Figure 2. Immunofluorescent labelling of transversal sections of cotyledons of *Copaifera langsdorffii* (A), *Tamarindus indica* (B) and *Tropaeolum majus* (C) with CCRC-M1 antibody. The arrowheads point to the labelling in the outer wall and the arrow points to the labelling in the inner wall. bar = 10 μm

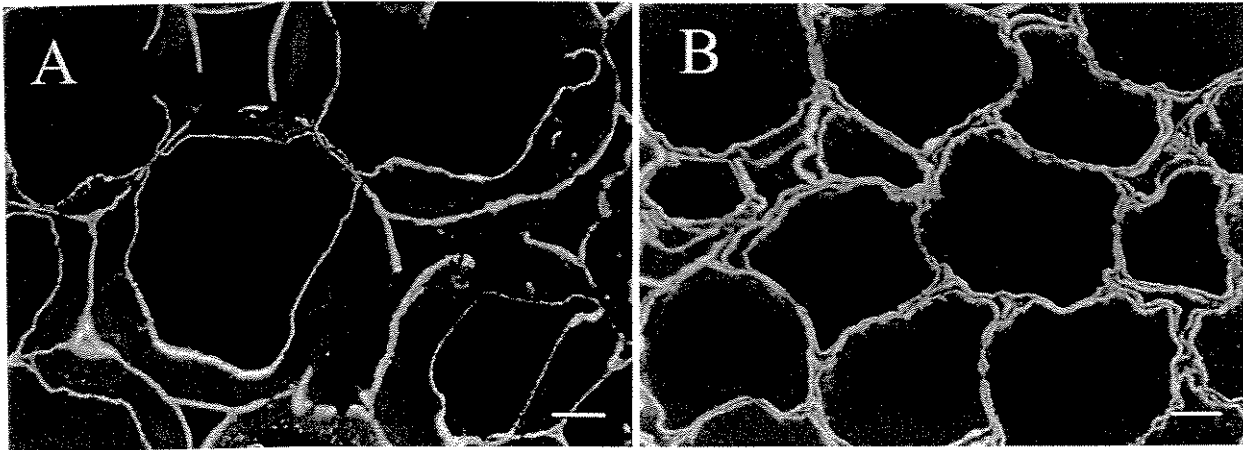


Figure 3. Immunofluorescent labelling of the fucosylated XG in sections of cotyledons of *Hymenaea courbaril* before (A) and after (B) mobilisation of the storage cell walls. Fluorescent walls were labelled with CCCR-M1 (first antibody) and FTIC (second antibody).X788; bar =10 μm .

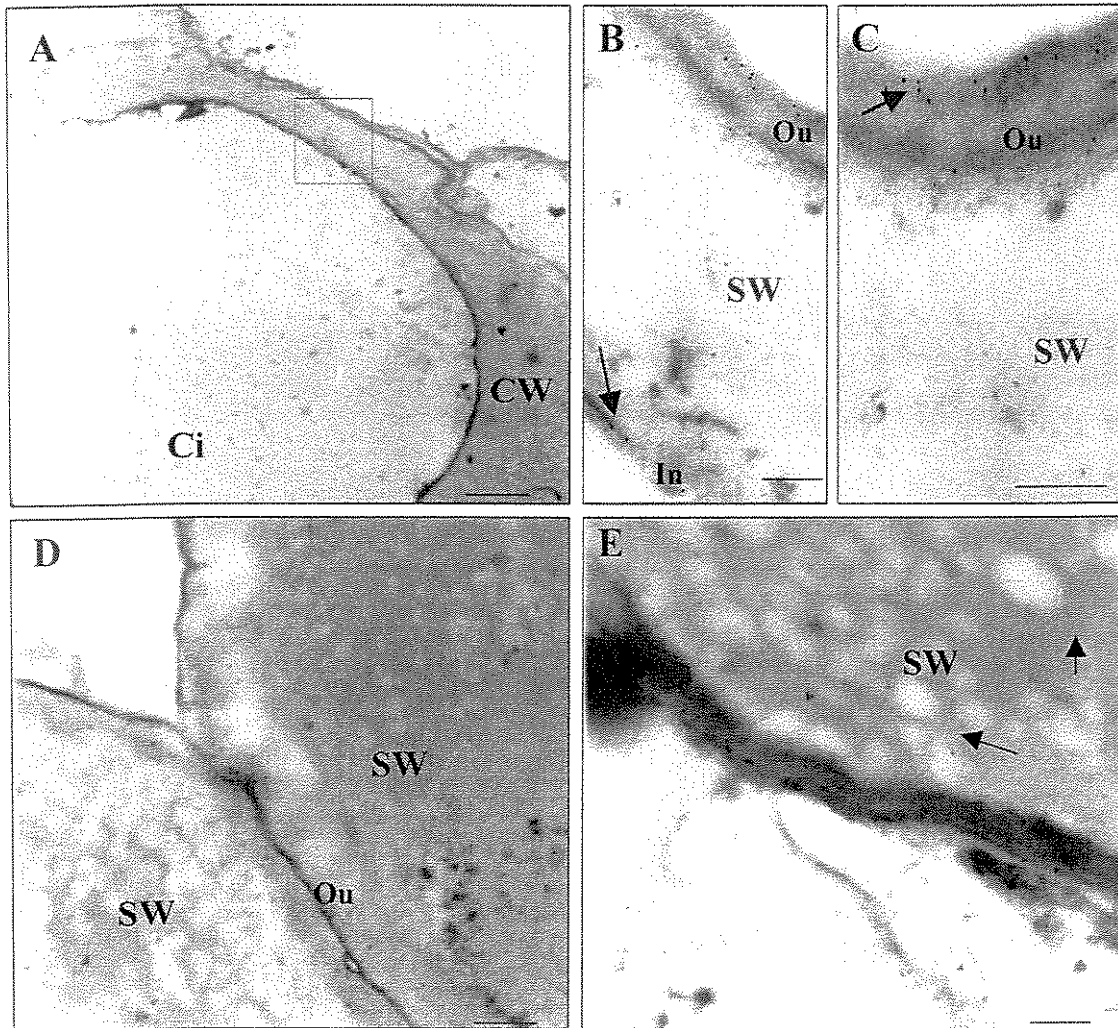


Figure 4. Immunogold labelling of the fucosylated XG in sections of cotyledons of *Hymenaea courbaril* by TEM. The box in A indicates the area enlarged in B. (A) Low magnification (2800X, bar = 2.5 μm). (B) High magnification (22000X, bar = 0.3 μm). (C) Detail of the outer layer of the wall. Arrows indicated the presence of fucosylated XG (gold particles) labelled with CCCR-M1 (first antibody). (D) Low magnification of the walls during XG degradation (2800X, bar = 2.5 μm). (E) High magnification of the walls during the mobilisation of the storage XG showing degradation pockets (arrow) in the storage wall (SW) (22000X, bar = 0.3 μm). Ci = cytoplasm, CW = cell wall, SW = storage XG; In = inner wall, Ou = outer wall.

Table II. *Linkage analysis of the storage polysaccharide of Hymenaea courbaril.*

Linkage	Proportion
t-Xyl	37.4
t-Gal	32.9
2-Xyl	5.7
4-Glc	5.3
4,6-Glc	18.6

Table II. *Monosaccharide composition of the fractions from Biogel P-6 chromatography. The fractions were identified as in figure 1. ND= not detected.*

Species	Biogel	Monosaccharides				
	Fractions	Glucose ^a	Xylose	Galactose	Fucose	Arabinose
<i>H. courbaril</i>	FI	4.00	2.50	1.30	0.4	0.08
	FII	4.00	2.30	2.3	0.4	0.13
	FIII	4.00	2.70	ND	ND	0.09
<i>C. langsdorffii</i>	FI	4.00	2.80	1.50	0.04	ND
	FII	4.00	2.80	1.60	0.08	0.01
	FIII	4.00	2.90	1.60	0.03	0.03
	FIV	4.00	2.80	1.70	0.02	0.02

^a The proportion of glucose was fixed as 4 residues according to the XG structure described for these species (Buckeridge et al., 1992; Tiné et al., 2000a).

Discussão

Estrutura do xiloglucano e ação das hidrolases

Neste trabalho foram caracterizados três novos oligossacarídeos presentes no xiloglucano de reserva de *Hymenaea courbaril* L. A existência de uma série de oligossacarídeos com cinco glucoses na cadeia principal havia sido relatada em 1997 nesta espécie (Buckeridge et al., 1997) e a ocorrência de XXXXG foi confirmada por espectrometria de massa e pela caracterização dos novos oligossacarídeos desta série que apresentam ramificações com galactose: XXXLG, XLXXG e XXLXG. A espectrometria de massa permitiu ainda determinar que ambas as séries de oligossacarídeos estão presentes em uma mesma molécula de xiloglucano e parecem estar alternadas ao longo do polímero.

Sabe-se que as substituições com xilose são capazes de impedir a ação da celulase sobre os resíduos glucosil substituídos. Em outro trabalho apresentado, foi possível mostrar que as substituições com galactosil são capazes de modular a interação entre a celulase e o xiloglucano, embora não sejam capazes de impedir completamente a ação da enzima. Como um comportamento semelhante foi encontrado tanto em *Hymenaea courbaril* quanto em *Copaifera langsdorffii* (que não possui a série de oligossacarídeos com cinco glucoses), é provável que este efeito seja independente do padrão de substituições com xilosil.

A localização na parede dos resíduos de fucose presentes no xiloglucano extraído dos cotilédones levou à caracterização de camadas na parede das células parenquimáticas. Ficou clara a separação das populações de xiloglucano que diferem não apenas na função, mas também na composição química e na localização na parede: um xiloglucano é estrutural e outro de reserva. Esta formação de camadas na parede implica em uma complexa coordenação do mecanismo de biossíntese e de deposição dos xiloglucanos durante a maturação da semente.

O uso de uma β -galactosidase já caracterizada no nosso laboratório permitiu a identificação de um dos novos oligossacarídeos que foi posteriormente corroborada pela espectrometria de massa. Apesar de se saber que esta β -galactosidase de *C. langsdorffii* só ataca o galactosil adjacente ao terminal não redutor do oligossacarídeo, não havia nenhuma evidência experimental de qual lado do substrato seria reconhecido pela enzima. Duas possibilidades haviam sido levantadas, a saber: o reconhecimento do substrato (oligossacarídeo) poderia ocorrer pelo terminal redutor ou pelo não redutor. Segundo o modelo corrente de degradação de XG de *H. courbaril* (Tin   et al., 2000), caso o reconhecimento ocorresse pelo terminal redutor, seriam necess  rias v  rias β -galactosidases para desmontar os oligossacar  deos, pois a cada ciclo de      da β -glucosidase e α -xilosidase, o pr  ximo galactosil ficaria “um monossacar  deo” (cerca de 0,5nm) mais pr  ximo do terminal redutor, devido    retirada da glucose. O modelo de reconhecimento pelo terminal n  o redutor, por outro lado, prev   a      de uma   nica β -galactosidase, uma vez que esta s   atuaria sobre o pr  ximo galactosil ap  s a remo    o do glucosil e do xilosil do terminal n  o redutor. Ap  s a      das outras exo-hidrolases, portanto, o galactosil estaria novamente    mesma dist  ncia do terminal n  o redutor que estava no ciclo anterior das hidrolases, embora estivesse 0,5nm mais pr  ximo do terminal redutor. Os dados obtidos neste trabalho apontam para o reconhecimento pelo terminal n  o redutor, pois a      da β -galactosidase sobre o XLXXG foi a mesma descrita para o XLXG (Alc  ntara et al., 1999).

Quanto ao reconhecimento pela celulase, seus s  tios de ataque ficam um res  duo glicosil mais distantes um do outro nos oligossacar  deos da s  rie do XXXXG. Na conforma    o cadeira do glucopiranos  deo, isso corresponde a cerca de 0,5 nm (Taylor e Atkins, 1985). Considerando que o s  tio catal  tico das celulases possui um tamanho semelhante (Figura 6), esta dist  ncia poderia deslocar o substrato para fora do s  tio catal  tico se o reconhecimento do substrato envolvesse o reconhecimento de dois poss  veis pontos simult  neos de ataque. Como a celulase    capaz de hidrolisar ambas as s  ries de oligossacar  deos, podemos concluir que, apesar de a cavidade catal  tica da enzima ser longa o suficiente para abranger oito res  duos de glucose, nem todos s  o

utilizados no reconhecimento do substrato. Esta flexibilidade no reconhecimento do substrato por parte da celulase é esperada quando consideramos que a enzima é capaz de hidrolisar o xiloglucano embora provavelmente tenha evoluído para hidrolisar as cadeias lineares de glucose da celulose onde não existem ramificações. Por outro lado, a redução dos fragmentos FII de *H. courbaril* com borohidreto diminuiu drasticamente a susceptibilidade à hidrólise com celulase e a taxa de formação de FI (dados não mostrados), o que sugere que mesmo alterações de conformação a quatro ou cinco resíduos de glucose do ponto de hidrólise são capazes de alterar a suscetibilidade dos fragmentos à celulase.

Além da diferença no comprimento do oligossacarídeo, a presença de oligossacarídeos com cinco glucoses na cadeia principal gera uma inversão desta que torna a molécula simétrica. Esta simetria não ocorre em xiloglucanos que não possuem oligossacarídeos com 5 glucoses em sua cadeia (Figura 7). No caso do xiloglucano apenas com 4 glucoses, um dos lados da molécula possui sítios de ataque pela celulase, enquanto o outro lado é altamente ramificado, o que não ocorre no xiloglucano que possui oligossacarídeos com 5 glucoses. Embora em solução a conformação da molécula seja dinâmica e o polissacarídeo possua pelo menos duas conformações de mínima energia (achatada e em espiral- Levy et al., 1991), esta mudança na simetria pode influenciar no reconhecimento do substrato pelas enzimas (XET e celulase).

A mudança na simetria proposta pode ser um importante elemento na interação com outros polissacarídeos. A experiência do nosso laboratório mostra que o xiloglucano de *H. courbaril* é mais difícil de solubilizar que os demais xiloglucanos de reserva (Miguel Minhoto, comunicação pessoal). O xiloglucano de *H. courbaril* se liga mais com a celulose que os demais xiloglucanos de reserva (Lima e Buckeridge, 2001). Se considerarmos a conformação achatada do xiloglucano proposta para a ligação com celulose (como representado na figura 7), esta adsorção maior poderia ser resultado da possibilidade de acesso a ambos os lados do xiloglucano, novamente como consequência da simetria do xiloglucano. Além disso, no caso de moléculas de xiloglucano associadas a outros polímeros, existe a possibilidade de ataque da celulase (ou XET) à face livre do xiloglucano, pois ambos os lados do polímero possuem sítios para a ação das enzimas.

Esta acessibilidade do polímero mesmo quando interagindo com outras moléculas pode ser um importante elemento em um sistema de reserva onde o xiloglucano precisa ser desmontado.

A existência de duas populações de xiloglucano química e espacialmente definidas na parede é um importante elemento na fisiologia da parede de reserva. Os dois tipos de xiloglucano possuem funções distintas claramente definidas: um é estrutural, menos solúvel, rico em arabinose (Tiné et al., 2000) e fucose, enquanto o outro é mais solúvel e composto apenas por glucose, xilose e galactose. Como as exo-hidrolases de xiloglucano só atacam resíduos não ramificados, a presença de substituições com fucose e arabinose deve bloquear a degradação de polímeros com estas substituições. Num sistema onde a degradação é limitada pelas exo-hidrolases, portanto, é natural que a ação da XET não seja um ponto de controle da degradação (Tiné et al., 2000).

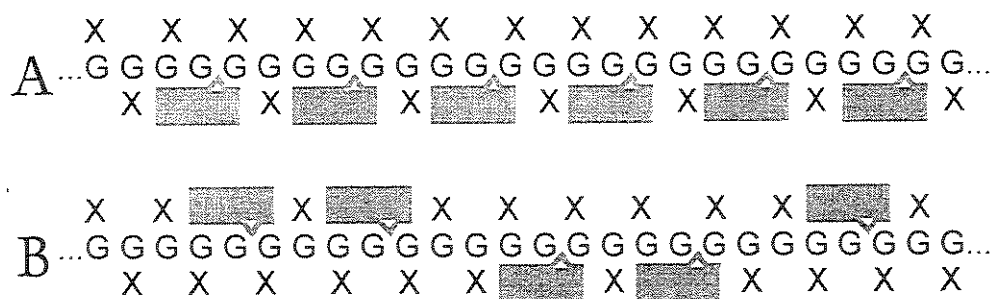


Figura 7. Comparação entre dois fragmentos de xiloglucano. A- xiloglucano composto apenas por oligossacarídeos com quatro glucoses na cadeia principal. B- xiloglucano com oligossacarídeos com quatro e cinco glucoses alternados. Os potenciais sítios de ataque da celulase estão indicados por barras nas moléculas. As galactoses não estão indicadas. Os blocos indicam a sequência de 3 glucoses requeridas para o reconhecimento do ponto de hidrólise na molécula. A posição hidrolisada está indicada pela cunha no bloco. É possível perceber a inversão da molécula em função da distribuição dos sítios de ataque da celulase (em B os sítios de ataque se alternam em ambos os lados da cadeia principal). G = glucose β -(1,4); X = xilose α -(1,6).

Evolução do xiloglucano de reserva em Leguminosae

Uma das principais características do xiloglucano é a sua capacidade de interagir com a celulose. A interação do xiloglucano com outras moléculas de xiloglucano, no entanto, passa a ser relevante em paredes de reserva onde a quantidade de celulose é menor e a densidade de xiloglucano é muito mais alta que na parede primária. Considerando a teoria de transferência de funções segundo a qual o xiloglucano de reserva teria evoluído a partir do xiloglucano de parede primária (Buckeridge et al., 2000a), um dos principais problemas a serem resolvidos ao longo da evolução da parede de reserva seria: como montar uma parede sólida com um mínimo de celulose?

Embora poucos xiloglucanos de reserva tenham sido caracterizados com detalhes, alguns padrões parecem emergir. Uma característica comum a todos eles é a remoção da fucose. Esta ramificação parece ocorrer exclusivamente em parede primária e sua remoção deve ser uma condição imprescindível para a montagem de uma parede de reserva. Considerando o modelo proposto para a biossíntese de xiloglucano, esta alteração envolveria uma modificação na fucosil-transferase na face trans do Complexo de Golgi. Não se sabe a razão desta necessidade de eliminação da fucose do polímero, mas além das alterações conformacionais induzidas pela fucose (Levy et al., 1991) já foi descrita atividade biológica dos oligossacarídeos de xiloglucano fucosilados (McDougall e Fry, 1988 e 1990, Emmerling e Seitz, 1990). A perda da fucose poderia ser uma forma de evitar o efeito biológico (antagônico à auxina) dos oligossacarídeos gerados durante a mobilização das reservas, separando os mecanismos de sinalização celular da degradação do xiloglucano. Uma outra alternativa seria a demarcação química entre duas populações de xiloglucano (de reserva e estrutural, como discutido anteriormente).

No que se refere à galactosil-transferase, no entanto, parece haver pelo menos duas estratégias. O xiloglucano de reserva de *T. majus*, *T. indica* e *C. langsdorffii*, quando submetido a hidrólise com celulase, produz uma mistura de oligossacarídeos rica em XLLG. As descrições de xiloglucano de parede primária da literatura, no entanto, mostram polímeros ricos em XXLG (e XXFG, o oligossacarídeo fucosilado derivado do XXLG). A abundância de motivos “XL...” em oligossacarídeos de xiloglucano parece,

portanto, estar associada a xiloglucano de reserva (Desveaux et al., 1998). Os oligossacarídeos de xiloglucano de *H. courbaril*, no entanto, são pobres em motivos “XL...”, mas possuem uma série de oligossacarídeos com cinco glucoses na cadeia principal.

As duas modificações no xiloglucano são funcionais, pois todas as espécies citadas conseguem se estabelecer em seus ambientes. Suas sementes germinam e o xiloglucano de reserva é mobilizado provavelmente por mecanismos muito semelhantes. Como estas duas estratégias são mutuamente exclusivas (rico em XLLG ou com XXXXG), aparentemente elas evoluíram para resolver o mesmo problema. Uma vez que uma das alternativas foi implementada, não há mais pressão de seleção para modificar o xiloglucano de reserva.

Nos estudos feitos em nosso laboratório com folhas de *H. courbaril*, a nova série de oligossacarídeos não foi detectada. Isso indica que a série de oligossacarídeos com cinco glucoses é utilizada exclusivamente para reserva nesta espécie, o que reforça a idéia de que são necessárias modificações no xiloglucano para que se possa montar uma parede de reserva rica neste polissacarídeo.

Codificação de informação no polímero

Em 1948, Claude Shanon publicou dois artigos que lançaram as bases para a teoria da informação (Shanon, 1948a,b). Esta teoria define informação do ponto de vista estatístico e, em termos gerais, confere a um símbolo qualquer uma quantidade de informação inversamente proporcional à frequência deste símbolo. Como informação é tratada de uma forma puramente estatística, esta teoria pode ser aplicada a qualquer seqüência, sejam números, letras, aminoácidos ou nucleotídeos. De fato, o avanço da bioinformática e a automação da análise de seqüências de DNA estenderam o uso desta teoria muito além do imaginado originalmente (Fleissner e Hofkirchner, 1996; Schneider, 2000). Atualmente, ferramentas de cálculo de frequência de nucleotídeos são capazes de estimar o número de diferentes proteínas que se ligam a um determinado domínio conservado baseando-se apenas na probabilidade com que uma seqüência

ocorre (Herman e Schneider, 1992). Basicamente, o que se sabe é que o tamanho de uma sequência conservada é apenas o necessário para que esta sequência seja identificada de forma inequívoca. Quando uma sequência possui o dobro da informação necessária para sua identificação, é provável que esta sequência seja reconhecida por dois ligantes independentemente.

Por analogia, podemos considerar que os padrões de ramificação de xiloglucano codificam informação (X, L ou G). Esta nova série de oligossacarídeos encontrada em *H. courbaril* representa uma forma distinta de distribuição das cadeias laterais e, portanto, aumenta o conteúdo informacional do xiloglucano. Como os sistemas biológicos geralmente não acrescentam mais informação do que o necessário para o desempenho das funções das moléculas, qual tipo de vantagem este aumento de informação traria? Algumas abordagens atuais consideram as enzimas como máquinas moleculares capazes de ler uma sequência de símbolos no substrato (nucleotídeos ou monossacarídeos) e calcular uma saída (catalizar reações) de acordo com o sinal lido no substrato. Em uma sequência com mais informação seria possível codificar novos comandos, aumentando o número de possibilidades na interação enzima-substrato.

Uma consequência direta desta alteração estrutural na interação com enzimas é a possível alteração nos mecanismos de controle. O modelo proposto para controle da degradação do xiloglucano de reserva coloca a β -galactosidase como a enzima responsável pelo “escape” dos oligossacarídeos do ciclo de transglicosilação (Ver Figura 5 e Tiné et al 2000). Como os oligossacarídeos de *H. courbaril* são relativamente pobres em extremidades “XL...”, a quantidade de substrato para a β -galactosidase é menor. Em ensaios de β -galactosidase no nosso laboratório, por exemplo, o uso desta mistura de oligossacarídeos como substrato levou a uma atividade muito baixa, provavelmente pela disponibilidade limitada de substrato, o que não ocorre quando usamos a mistura de oligossacarídeos de *C. langsdorffii*, rica em XLLG (Clóvis Oliveira Silva, comunicação pessoal). A enzima responsável pela remoção dos fragmentos de xiloglucano do ciclo da XET no caso de *H. courbaril*, portanto, deve ser a α -xilosidase (Figura 5). O modelo proposto anteriormente, no entanto, continua sendo válido para as espécies ricas em XLLG.

Mesmo com esta alteração da ordem em que as enzimas atuam sobre os oligossacarídeos de *H. courbaril*, a β -galactosidase continua sendo provavelmente um dos pontos de controle da degradação do xiloglucano. Como as exo-hidrolases se alternam em um ciclo para desmontar um polímero, a redução de atividade de qualquer uma das hidrolases (α -xilosidase, β -galactosidase e β -glucosidase) poderia levar a uma redução no ritmo de degradação. A diferença de pH ótimo das enzimas, portanto, continua sendo um provável elemento de controle. Como existe uma diferença no pH ótimo destas enzimas (pH ótimo em torno de 3,5 para a β -galactosidase e 4,5 para as outras duas exo-hidrolases), oscilações no pH da parede poderiam modular a ação das enzimas sobre os oligossacarídeos da parede (Tin   et al., 2000). A ordem em que as enzimas ir  o atuar no pol  mero, no entanto, depende da estrutura fina deste. Assim, parte da informa  o para a degrada  o do pol  mero    codificada no pr  prio substrato j   no momento de sua bioss  ntese durante a maturac  o da semente.

No caso dos polissacar  deos de *T. majus*, *C. langsdorffii* e *T. indica*, a passagem de xiloglucano de parede prim  ria para xiloglucano de reserva durante a evolu  o parece estar relacionada essencialmente a alterac  es na atividade de galactosil- e fucosil-transferases. No caso do polissacar  deo de *H. courbaril*, parece ter havido uma alterac  o do local onde a informa  o    codificada, pois j   na face cis do complexo de Golgi a bioss  ntese de blocos de 4 ou 5 glucoses cria um padr  o diferente de ramifica  es, enquanto a atividade de galactosil-transferase n  o parece ser muito modificada. Os processos de degrada  o e bioss  ntese do xiloglucano de reserva, portanto, parecem estar finamente sincronizados nas esp  cies e o ve  culo para a integra  o entre os dois processos pode ser o pr  prio polissacar  deo, uma vez que o padr  o de ramifica  o do xiloglucano    capaz de codificar informa  o suficiente para modular a a  o das enzimas.

Al  m da codifica  o de informa  o atrav  s da seq  ncia do pol  mero, existe a possibilidade de a organiza  o da parede trazer informa  es topol  gicas (relativas    forma). Se, por exemplo, virarmos uma membrana qualquer dentro da c  lula pelo avesso, dificilmente esta membrana continuar   exercendo corretamente suas fun  es, apesar de nenhuma alterac  o de composi  o ter sido provocada. Isso porque a organiza  o espacial dos elementos celulares cont  m, em si mesmo, informa  o

necessária ao correto funcionamento da célula. Neste contexto, a localização da camada de xiloglucano de reserva entre duas camadas estruturais, pode trazer um importante elemento na compreensão da fisiologia da parede celular de reserva. Estes conceitos de informação mais abrangentes podem trazer novos pontos de vista sobre a bioquímica e possibilitar a integração de diversos elementos da fisiologia: composição química, enzimologia e localização.

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