l'université nantes angers le mans
PÔLE DE RECHERCHE ET D'ENSEIGNEMENT SUPÉRIEUR

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Fanny Buffetto

Mémoire présenté en vue de l'obtention du
grade de Docteur de l'Université de Nantes
sous le label de L'Université Nantes-Angers-Le Mans

École doctorale : Végétal, Environnement, Nutrition, Agro-alimentaire, Mer (VENAM)
Discipline : Aspect moléculaire et cellulaire de la biologie
Spécialité : Glycanes de la paroi des cellules végétales
Unité de recherche : Biopolymères, interactions, assemblages (INRA Angers-Nantes)

Generation of new monoclonal antibodies to rhamnogalacturonan fragments

JURY

Rapporteurs :	**Henk SCHOLS**, Professor, University of Wageningen
	Pierre VAN CUTSEM, Professor, University of Namur
Examinateurs :	**Patrice LEROUGE**, Professor, University of Rouen
	Philipe SIMIER, Professor, University of Nantes
Directeur de Thèse :	**Fabienne GUILLON**, DR, INRA Nantes
Co-directeur de Thèse :	**Marie-Christine RALET**, DR, INRA Nantes

Generation of new monoclonal antibodies to rhamnogalacturonan fragments

Fanny BUFFETTO

Thesis commitee

Board of examiner: **Henk SCHOLS**, Professor, University of Wageningen
Pierre VAN CUTSEM, Professor, University of Namur

Reviewer : **Patrice LEROUGE**, Professor, University of Rouen
Philippe SIMIER, Professor, University of Nantes

Thesis supervisor : **Fabienne GUILLON**, DR, INRA Nantes

Thesis co-supervisor : **Marie-Christine RALET**, DR, INRA Nantes

Remerciements

Ce travail de thèse a été réalisé à l'INRA de Nantes au sein de l'unité Biopolymères, Interactions, Assemblages (BIA) sous la direction de Fabienne Guillon et Marie-Christine Ralet avec le soutien financier de l'union Européenne (FP7 2007-2013).

Je remercie tout d'abord Fabienne Guillon qui m'a donné l'opportunité d'acquérir tous ces savoirs, ces trois dernières années et de m'avoir transmis son énergie et sa passion pour la recherche. Je remercie Marie-Christine Ralet pour son enthousiasme permanent, sa disponibilité sans mesure et son encouragement. Je remercie Marie-Jeanne Crépeau pour sa gentillesse d'avoir partagé avec moi tant de ses connaissances. Je remercie Alexandre Thebaud pour avoir toujours été à l'écoute et d'avoir pris tant soin de tous ces petits «fellows». Je remercie David Ropatz et Audrey Geairon de m'avoir aidé à décortiquer ces petits fragments de rhamnogalacturonan parfois si coriaces. Je remercie Olivier Tranquet, Valérie Echasserieau et Julie Leluhant de m'avoir appris l'élevage intensif de petites cellules productrices d'anticorps. Je remercie Camille Alvarado et Brigitte Bouchet pour leur aide précieuse en microscopie. Je remercie Jacqueline Vigouroux et Sophie Le Gall pour leur initiation aux perméthylations et de m'avoir remonté le moral après « l'histoire du tube en plastique ». Je remercie Paul Robert et Sylvie Durand qui ont partagé avec moi leur collection de spectres de pectine que je n'ai malheureusement pas pu tant compléter. Je remercie Adelin Barbacci pour ses macros qui m'ont énormément servis en spectrométrie de masse. Je remercie Laurent Helary pour avoir toujours su dédramatiser mes relations avec mon ordinateur. Je remercie Luc Saulnier d'avoir subi quelques-unes de ces mésententes avec ce même ordinateur et d'avoir bercé toute ma dernière année de thèse avec sa sonnerie de bateau. ☺

Je remercie en général toute l'équipe PVPP pour votre bonne humeur : J'espère vraiment retrouver une ambiance d'équipe comme la vôtre.

Je remercie William Willats de m'avoir reçu dans son laboratoire à Copenhague. Sous le soleil danois, j'ai été merveilleusement accueillie par toute son Equipe et Maja. Je remercie également Frank Meulewaeter de m'avoir reçu dans son laboratoire à Bayer. Je remercie Nadine et Jean-Luc d'avoir collaboré à la réalisation de PACEs en série régis sous un système de production proche du fordisme. Aussi, je voudrais remercier toutes les personnes impliquées dans le projet WalltraC avec qui j'ai pu partager des moments inoubliables dans les quatre coins de l'Europe.

Je remercie Mr Philippe Simier professeur de l'université de Nantes d'avoir accepté de présider ce jury de thèse. Je remercie également Mr Henk Schols, Professeur de l'université de Wageningen et Mr Pierre Van Cutsem, Professeur de l'université de Namur, d'avoir gentiment accepté d'être rapporteurs de ce travail. Je remercie Patrice Lerouge, Professeur de l'université de Rouen, d'avoir accepté d'examiner ce travail.

Merci

Je voudrais, de même, remercier toutes les personnes qui ont été contraintes et forcées de partager ce moment avec moi :

Ma première pensée vient à ma copine de « chambre » Amel qui m'a dopée tout au long de ma deuxième moitié de thèse avec sa joie de vivre et son chocolat. Puis, à toute la petite troupe féminine de PVPP : Sayani, Séverine, Merve, Emmanuelle, Nadia, Fatma, Pauline, Florianne. Une autre pensée pour Valérie de Leeds avec qui j'ai partagé des moments magiques depuis notre première rencontre dans la gare de Copenhague.

Je voudrais également faire un big up à toutes les racailles de la Motte Rouge (Maturin la Star, Clément le militant, l'épatante Gallou, Vio la bimbo, l'attachant Wayna, le bouillonnant J-lou , l'attentionnée Glad, Jb le tailleur de gazon, Iwan le maitre du jeu, le raffiné Marceau, l'(h)ardente Marion, le gargantuesque Jash, l'acide Benj, La belle Elo, Pak l'expat', La reine des reines K-ren, the MC Mijer, Tristan l'ourson et la grasse-ieuse Mathieu) ainsi qu'à Bichette pour son acharnement et son éclatante victoire contre Word2010 (Bichette vs Word :3-2), à la féerique petite Léa, à Rhinosouris l'incroyable créature qui déborde d'imagination, de drôlerie et de bonne humeur, ainsi qu'aux supers bretons de Guingamp avec une pensée toute particulière pour mon Cyril… ☺ We are one we are love ☺

Je ne pourrai enfin, terminer ce texte sans remercier la Famiglia Buffetto avec le généreux Nico, la rayonnante Kiki, et Remy le vrai père fondateur de la doctrine épicurienne ☺, ainsi que Les deux sœurs inséparables, les deux petits poulets, les deux jeunes parents, les deux champions de Bridge, Les deux globes trotteurs et les grandes sœurs auvergnates…

Diantre merci

TABLE OF CONTENTS

CHAPTER III

List of Figures and Tables

Chapter I

Chapter II

Chapter III

Chapter IV

Abreviations

AEC: anion-exchange chromatography

AGP: arbinogalactan protein

Ara-BSA: arabinan derived oligosaccharide coupled with BSA

ARAD1: gene encoding for a putative arabinosyltransferase

arad1: mutant for the genes ARAD1

BCIP: 5-bromo,4-chloro,3-indolyphosphatase

BSA: bovine serum albumin

BT-PBS: PBS containing 1 % BSA and 0.05 % Tween

Ca: calcium element

Ca^{2+}: calcium ion

CESA: cellulose synthase

DA: degree of acetylation

DE: degree of esterification

DEAE: diethylaminoethanol

DM: degree of methylation

DMSO: dimethylsulfoxyde

DP: degree of polymerisation

ELISA: enzyme linked immunosorbent assay

FID: flame ionization detector

FT-IR: Fourier transform infrared

Gal-BSA: galactan derived oligosaccharide coupled with BSA

GALS1, GALS2, GALS3: genes encoding for 1,4- β-D-galactose-transferases

gals1, gals2, gals3: mutant for the genes GALS1, GALS2, GALS3

GAUT1: gene encoding for a GAlactUronyl-Transferase

GAUT7: gene encoding for a protein which is complexed with GAUT1 and is anchored to the plasma membrane

GC/MS: gas chromatography coupled to mass spectrometry

GC: gas chromatography

GRP: glycyne rich protein

GT: glysosyl-transferase

GUT1: gene encoding for a putative glucurosyl-transferase

HG: homogalacturonan

HM: high methoxy-pectin

HRGP: hydroxyproline protein

Hyp: hydroxyproline

IC50: is the concentration of inhibitor required to inhibit 50% of the mAb bounding to the antigene coated to the plate

IgG: immunoglobulin G

IgM: immunoglobulin M (pentameric)

irx10/irx10l: double mutants for gene IRX10/IRX10L (orthologous gene of GUT1)

KNU: unit which corresponds to the amount of enzyme which hydrolyses 4870 mg of starch per hour

LC-MS: liquid chromatography coupled to MS

LM: low methoxy-pectin

Lys: lysine

mAb: monoclonal antibody

MES: 2(N-morpholino)ethane sulfonic acid

Mg^{2+}: magnesium ion

Mn^{2+}: manganese ion

MS/MS: tandem MS

MS: mass spectrometry

NBT: nitroblue tetrazolium

NMR: nuclear magnetic resonance

nolac-H18: GUT1mutant

OD: optical density

OVA: ovalbumin

PBS: phosphate buffered saline

PGC-LC: porous graphitized carbon liquid chromatography

PGIP: polygalacturonase inhibitor protein

Pro: proline

PRP: proline rich protein

QUA1/GAUT8: putative gene encoding for a Galacturonyl-Transferase

QUA2 and QUA3: putative genes encoding for pectin methyl-transferases

RGI: rhamnogalacturonan I

RGII: rhamnogalacturonan II

Rgxt1; Rgxt2; Rgxt3; Rgxt4: mutant for the RGXT1; RGXT2; RGXT3; RGXT4 genes

RGXT1; RGXT1; RGXT1; RGXT4: gene encoding for 1,3-α-D-xylosyltransferases

RU: oligosaccharides generated from RGI unbranched backbone

RUB: RGI oligosaccharides extracted from sugar beet pulp

RU-BSA: RU derived oligosaccharide coupled with BSA

RUP: RGI oligosaccharides extracted from potato pulp

RUP-BSA: RUP oligosaccharides coupled to BSA

RUP-OVA: RUP oligosaccharides coupled to OVA

SEC: size exclusion chromatography

Ser: serine

T-DNA: DNA transfert

TFA: trifluoroacetic acid

TMSV: total mean sample value

TOF: time-of-flight

Tyr: tyrosine

UI: international unit

Val: valine

xgd1: mutant for the gene XGD1

XGD1: xylogalacturonan deficient 1 gene

Monosaccharides conventional abbreviations:

Ara: arabinose

DHA: 3-desoxy-D-manno-2-octulosonic acid

Fuc: fucose

Gal: galactose

GalA: galacturonic acid

Glc: glucose

GlcA: glucuronic acid

KDO: 3-desoxy-D-lyxo-2-heptulosonic acid

Man: mannose

Rha: rhamnose

Xyl: xylose

f: furanose form

p: pyranose form

Résumé en français

Production d'anticorps monoclonaux originaux à partir de fragments de rhamnogalacturonanes

Introduction

Cette thèse a été initiée dans le cadre du projet européen WallTraC. Ce projet est un ITN (initial training network) Marie Curie ayant deux objectifs majeurs:

-Le développement de nouveaux outils moléculaires et de techniques pour l'analyse de la paroi végétale. La cellulose et les pectines sont les principales cibles de ce projet étant donné leur large utilisation dans le secteur industriel en tant qu'ingrédients fonctionnels alimentaires et fibres textiles.

-La formation de jeunes chercheurs dans le domaine de la paroi végétale à travers la mise en place de formations transdisciplinaires, intersectorielles et de haute qualité sur l'analyse de la paroi cellulaire ainsi que sur la conduite de projets de recherche.

Au sein du projet WalltraC, ma thèse a pour but le développement de nouveaux anticorps (Ac) permettant la localisation de structures spécifiques de pectines *in planta*. Des Acs reconnaissant les pectines ont déjà été développés. Néanmoins, pris tous ensemble, ils ne permettent pas de recouvrir la complexité de ce polymère.

Etat de l'art (chapitre I)

La paroi végétale est un élément de structure qui protège chaque cellule des plantes. Elle est organisée en trois parties distinctes : la paroi primaire, la paroi secondaire et la lamelle moyenne. La paroi primaire des eudicotylédones et la lamelle moyenne sont les entités majoritairement concentrées en pectines. Schématiquement la paroi cellulaire est édifiée de fibres de cellulose enchâssées dans une matrice composée de protéines ainsi que de pectines. La lamelle moyenne est majoritairement constituée de pectines.

Les pectines représentent 1/3 de la paroi primaire des eudicotylédones. Ce sont des polymères acides de par les fonctions carboxyliques présentes sur les acides galacturoniques. Différents domaines sont constitutifs des pectines (Figure.A). Les homogalacturonanes (HG) sont des polysaccharides linéaires d'acides galacturoniques. Les analogues d'homogalacturonane contiennent tous une chaîne principale d'HG. Les apiogalacturonanes et xylogalacturonanes contiennent respectivement des résidus d'apiose et de xylose qui sont branchés sur les acides galacturoniques. Le rhamnogalacturonane II (RGII) est un domaine complexe constitué de nombreux sucres rares contenus dans 5 chaînes latérales. Le rhamnogalacturonane I (RGI) est la seule structure pectique édifiée sur un squelette hétérogène d'acides galacturoniques et de rhamnoses. Ce domaine peut être branché par des chaînes latérales comprenant majoritairement des arabinoses et des galactoses. Tous ces domaines pectiques sont liés directement de façon covalente ou indirectement par association au calcium, au bore ou à l'acide férulique. Des liaisons covalentes entre les pectines et hémicelluloses ou protéines structurales ont également été décrites.

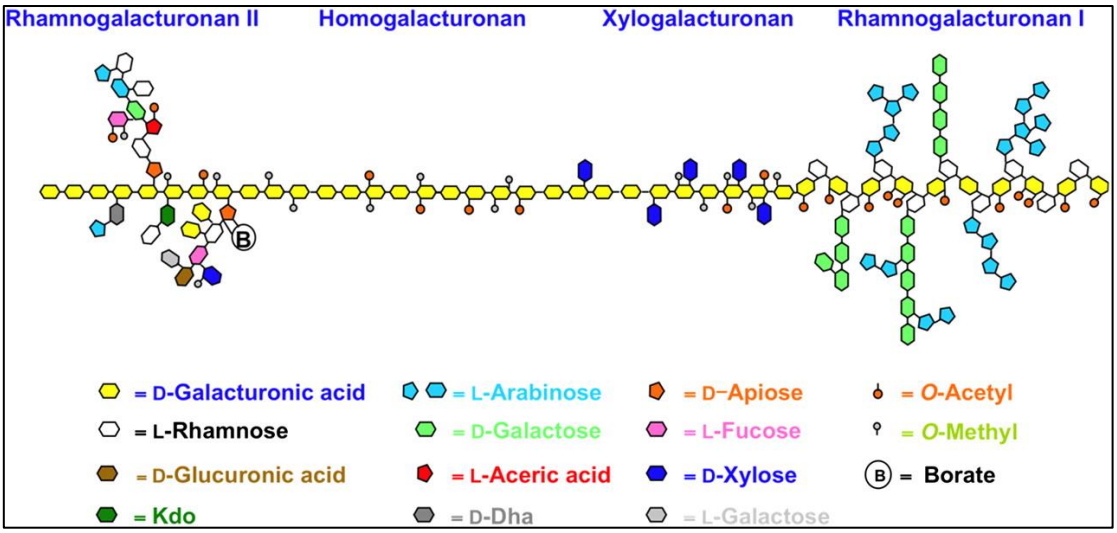


Figure A : Structure des pectines (Harholt J. Suttangkakul A. Scheller H.V. 2010. biosynthesis of pectin plant physiology, 153: 384–395)

La biosynthèse et la fonction des pectines ne sont encore pas concrètement définies. Néanmoins, certaines études ont permis une avancée majeure dans la compréhension du rôle de ce polymère. Seules quelques enzymes impliquées dans le métabolisme des pectines ont été caractérisées. Le problème de redondance de ces enzymes engagées dans la biosynthèse, ne permet pas d'évaluer l'impact réel de leur mutation sur le phénotype de la plante transformée. Différentes fonctions ont malgré tout été suggérées, proposant les pectines comme principaux texturants de la paroi primaire des dicotylédones. Les différentes structures d'HG (méthylés, déméthylés, dimerisés) ont été identifiées comme ayant un rôle majeur dans l'élongation des cellules, le mûrissement des fruits ou encore la signalisation cellulaire. Les dimérisations des HG et des RGII contribuent largement à l'adhésion des cellules entre elles. Les chaînes latérales d'oses neutres des RGI plus flexibles, permettent de maintenir la matrice extracellulaire hydratée, tout en ayant un rôle structurant. Les nombreuses chaînes latérales des RGI présentes dans les parois des cotylédons des graines suggèrent également que ces domaines pectiques peuvent être assimilés à des molécules de réserve.

Stratégie et objectifs de la thèse

Le développement de nouveaux anticorps est nécessaire pour l'analyse de structures pectiques dans les parois cellulaires des plantes. Le rhamnogalacturonane I et le rhamnogalacturonane II ont été les domaines cibles en raison de leur diversité structurale et d'une connaissance très sommaire de leurs fonctions *in planta*. Les anticorps disponibles contre le rhamnogalacturonane I sont principalement spécifiques des structures linéaires du squelette principal et des chaînes latérales, ce qui ne reflète pas la grande hétérogénéité de cette molécule. Contrairement au rhamnogalacturonane I, aucun anticorps monoclonal n'a été produit contre le domaine rhamnogalacturonane II. Différentes étapes sont nécessaires à la production d'anticorps monoclonaux. Tout d'abord, les glycanes contenant la région qui sera ciblée par l'anticorps doivent être préparés. Dans un second temps, les fragments glycosidiques sont injectés à l'animal. Enfin, les anticorps d'intérêt sont sélectionnés pour être caractérisés afin de déterminer la structure, reconnue par les nouvelles sondes produites.

Les domaines de rhamnogalacturonane I et les rhamnogalacturonane II ont été extraits de sources végétales diverses (Figure.B). A partir de ces domaines, des oligosaccharides très spécifiques ont été purifiés. Leur production a été réalisée à partir de différentes sources de plantes, par le biais de diverses hydrolyses chimiques et enzymatiques. Ces oligosaccharides ont été purifiés en utilisant principalement des techniques de chromatographie basse pression. Les oligosaccharides issus de RGI ont été purifiés à partir de pomme de terre (RUP) et de betterave (RUB). Ces deux sources ont été sélectionnées car elles diffèrent par leur composition en oses (pomme de terre riche en galactane et betterave riche en arabinane). Le recours à des hydrolyses acides ménagées et à des hydrolyses enzymatiques a permis de réduire la plupart des chaînes latérales et principales linéaires. Les oligosaccharides résultants sont supposés très substitués et enrichis en motifs ciblés pour la reconnaissance des mAbs, contenant les points de jonctions entre les chaînes latérales et la chaîne principale. Les oligosaccharides de RGII ont été purifiés à partir de vin. Ce petit domaine pectique est branché par cinq chaînes latérales (A-E) liées sur un squelette homogalacturonique. Pour éviter de produire des anticorps reconnaissant la chaîne

principale d'acides galacturoniques, il a été décidé de purifier les chaînes latérales A et B. Leur structure est connue pour être largement conservée au cours de l'évolution. Tous ces oligosaccharides ont été produits en collaboration avec deux autres doctorants du projet WallTraC : Valérie Cornuault (Université de Leeds, UK) a généré les oligosaccharides dérivés de RGI de pomme de terre et Jeff Zhang (Université de Newcastle, UK) a quant à lui pris soin de l'extraction et de la purification des RGII. Leurs travaux ont tous deux été réalisés à l'INRA de Nantes sous la supervision de Marie-Christine Ralet. Les fragments pectiques ont été structurellement caractérisés en utilisant différentes méthodes de biochimie analytique et de spectrométrie de masse.

Les quatre pools d'oligosaccharides (RUP, RUB, chaînes latérales A et chaînes latérales B) ont été couplés à une protéine immunogène. Les néoglycoproteines ont été injectées à des souris (3 souris par néoglycoprotéine) de façon à déclencher la production de différents anticorps reconnaissant des structures spécifiques de RGI et RGII. Les lymphocytes des souris ont été extraits, puis hybridés avec des cellules cancéreuses immortelles. Des hybridomes produisant des anticorps reconnaissant les oligosaccharides RUP ont été sélectionnés. Malheureusement, les immunisations réalisées avec les oligosaccharides RUB et les chaînes latérales A et B n'ont pas permis la production d'anticorps reconnaissant les RGI de betterave et les RGII. Les anticorps prometteurs reconnaissant le pool d'oligosaccharides RUP ont été caractérisés par une méthode d'analyse à haut débit, la technique d'impression de glycanes sur plaques de nitrocellulose, mise en place à l'Université de Copenhague par le professeur William Willats. Les anticorps présentaient tous un profil de reconnaissance très semblable vis-à-vis des polysaccharides et des oligosaccharides testés. Suite à cette expérience un seul anticorps a été sélectionné pour la caractérisation plus fine de l'épitope reconnu. Cet anticorps a également été utilisé en microscopie par immunofluorescence sur plusieurs espèces végétales.

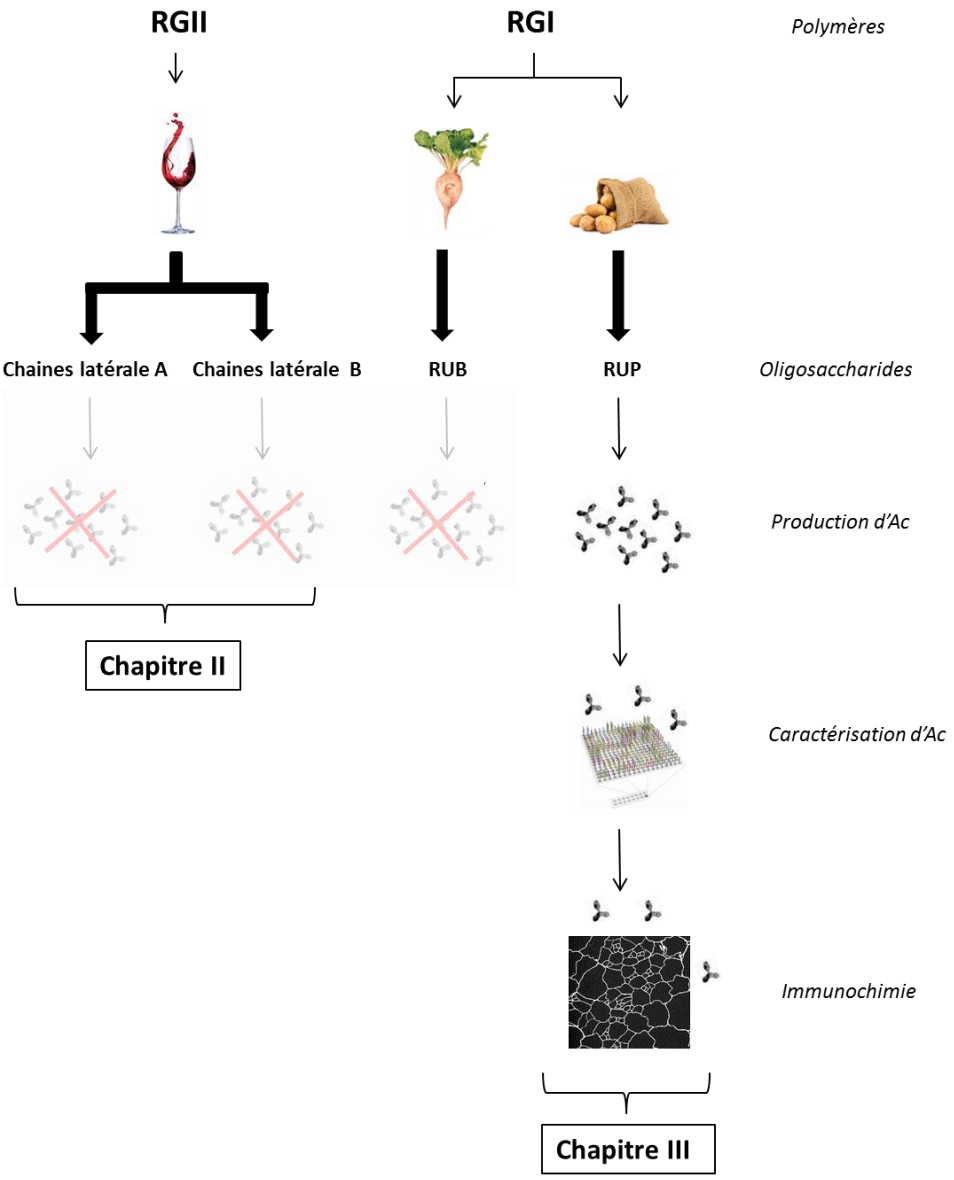


Figure B: Stratégie suivie et résultats développés dans les chapitres I et II

Dans la partie contenant les résultats, le premier chapitre relate le travail effectué pour l'isolement des chaînes latérales A et B du RGII. Le recouvrement des chaînes latérales est décrit ainsi que leur caractérisation et celle de la chaîne principale d'acides galacturoniques. La variabilité de la structure du RGII a été étudiée après hydrolyses douces et séquentielles. Le chapitre II traite de l'isolement et de la caractérisation des oligosaccharides RUP, ainsi que de la production et de la caractérisation pour l'utilisation cytochimique, des nouveaux anticorps monoclonaux reconnaissant ces oligosaccharides.

Résultats

Partie 1 (chapitre II) : Purification de sous-domaines de rhamnogalacturonanes II et description de la variabilité des structures caractérisées.

Le RGII II est un domaine pectique composé de plus de 12 différents oses et 20 différentes liaisons. Cinq chaînes latérales sont distribuées sur une chaîne principale d'acides galacturoniques. Cette structure a longtemps été considérée comme très conservée tout au long de l'évolution du monde végétal. De récentes études sur la caractérisation de ce domaine ont néanmoins révélé la présence de variations structurales. L'étude décrite dans ce chapitre rapporte les diverses structures constitutives du RGII extrait de vin. D'importantes variations ont été retrouvées dans les structures de la chaîne principale et des chaînes latérales A et B. Ces chaînes secondaires ont été obtenues par hydrolyses acides douces et séquentielles. Cette étude a été réalisée pour mettre en exergue les variations intrinsèques des chaînes latérales du RGII.

Pour cela, le RGII de vin a été hydrolysé avec de l'acide trifluoroacetique (TFA) et les produits d'hydrolyse ont été séparés en chromatographie échange d'anions. Les fractions issues de la séparation chromatographique ainsi que certains hydrolysats non fractionnés ont été analysés par spectrométrie de masse (MALDI-TOF). Les conditions optimales d'hydrolyse ont été définies pour l'obtention de chaînes latérales B (TFA 0.1 M ; 40 °C ; 8 h) et de chaînes latérales A (TFA 0.48 M ; 40 °C ; 8 h ou TFA 0.1 M ; 40 °C ; 8 h) non dégradées. Les conditions définies pour obtenir le squelette principal de HG ont nécessité une incubation à 60 °C avec du TFA à 0.48 M pendant 16 h. La séparation chromatographique a permis l'isolement de la chaîne B, des chaînes A méthylestérifiées et non-méthylestérifiées. Une grande variabilité de la structure de la chaîne A a pu être mise en évidence par analyse en spectrométrie de masse.

En conclusion, de nombreuses variations ont été observées dans la structure du RGII. La présence variable d'arabinoses terminaux et de groupements acétyles a été identifiée

comme étant généralement la conséquence des traitements acides, alors que les methyl-estérifications et éthérification, ainsi que l'oxydation de la chaîne A, sont plutôt le reflet de la diversité naturelle. La signification physiologique de cette variabilité reste encore à déterminer.

Partie 2 (chapitre III): Nouvel anticorps produit contre les chaînes latérales de RGI

Le rhamnogalacturonane I est un domaine pectique présentant une haute variabilité. 20 à 80% des rhamnoses peuvent être branchés par des chaînes latérales composées majoritairement d'arabinoses et de galactoses. La variabilité de la composition des chaînes latérales dépend des organes considérés, du type de plante, ou encore de la morphogénèse. Pour localiser *in planta* les structures de RGI, des anticorps ont été produits. LM5 et LM9 reconnaissent des structures de galactanes. LM6, LM13 et LM16 se fixent sur des chaînes d'arabinanes. Le squelette principal de rhamnogalacturonane non branché est reconnu par INRA-RU1 et INRA-RU2. Cependant, peu d'anticorps reconnaissent des structures branchées et complexes de RGI. Ce chapitre décrit donc la génération d'un nouvel anticorps spécifique d'un sous-domaine particulier de RGI.

La première étape de ce travail a consisté à produire et purifier des oligosaccharides de RGI, à partir de tubercules de pomme de terre. Les RGI ont été extraits de pulpe de pomme de terre commerciale, par hydrolyse alcaline à haute température. Dans ces conditions, par beta-élimination, les HG sont simultanément deesterifiés et dépolymérisés. Les RGI ont ensuite été séparés des fragments de HG par chromatographie d'échange d'anions. Différentes hydrolyses enzymatiques ont ensuite été appliquées, pour dégrader partiellement les chaînes latérales linéaires de galactanes et d'arabinanes, puis la chaîne principale non branchée. Les fragments produits (appelés oligosaccharides RUP) ont ensuite été recouvrés par extraction à l'éthanol 50%. Les oligosaccharides RUP ainsi produits ont été couplés à une protéine immunogène pour aider au déclenchement de la réponse immunitaire des souris. Les anticorps secrétés par les lymphocytes se fixant sur les oligosaccharides RUP ont été sélectionnés pour la caractérisation fine de l'épitope reconnu.

L'utilisation de plaques de nitrocellulose à haute densité a permis la sélection d'un clone produisant un nouvel anticorps. L'épitope a été caractérisé plus finement par séparation des oligosaccharides RUP en chromatographie échange d'anions, suivi par des tests d'inhibition de chacune des fractions en ELISA. Les fractions considérées ont été ultérieurement analysées par perméthylation et spéctrométrie de masse. L'anticorps a été également utilisé en microscopie sur des racines natives d'Arabidopsis et sur coupes de pomme de terre (Figure.C).

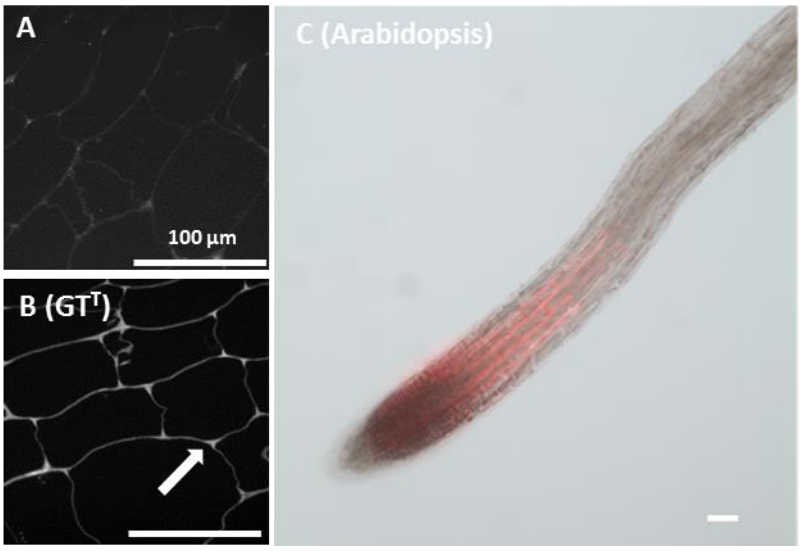


Figure C : Immunomarquage de RUP2. Coupe du periderme de pomme de terre (A et B). Le marquage de RUP2 est absent sans traitement enzymatique (A) mais l'épitope de RUP2 est révélé (B) après traitement à la galactanase (GT^T). Il est notament très présent dans les zones de jonction des cellules (flèche). Sans besoin de traitement enzymatique, le marquage de RUP2 est plutôt situé au niveau de la zone méristematique et de la zone d'élongation des racines des jeunes pousses d'Arabidopsis (C).

Le clone RUP2 a pu être identifié comme producteur d'un nouvel anticorps du même nom, reconnaissant les RGI. L'analyse des structures présentes dans le pool d'oligosaccharides RUP et reconnues par l'anticorps, indique que ce dernier est spécifique d'une structure linéaire composée d'arabinoses et de galactoses. Ce motif est présent dans les parois des végétaux riches en galactanes. Dans la zone périphérique du tubercule de pomme de terre, l'épitope est mis en évidence après élimination des chaînes de galactanes, mais aussi d'arabinanes linéaires. Dans les racines d'*Arabidopsis thaliana*, cette chaîne mixte a été observée sans besoin de pré-traitement, au niveau de la zone d'élongation, une région riche en galactose.

Un nouvel anticorps spécifique des chaînes latérales de RGI a été généré. Malgré la purification de structures branchées de RGI pour l'immunisation, cet anticorps reconnait une structure linéaire de chaînes latérales contenant à la fois des galactoses et des arabinoses. RUP2 se fixe sur les RGI de plantes riches en galactanes.

Conclusion et perspectives (chapitre IV)

Les rhamnogalacturonanes sont les domaines pectiques suscitant beaucoup d'interrogations concernant la régulation de leur synthèse et leurs fonctions *in planta*. La microscopie par immunolocalisation est une technique très sensible pour la détection de structures dans les tissus végétaux. Cependant, les anticorps reconnaissant les pectines ne sont pas assez nombreux pour permettre une cartographie de la localisation de tous les domaines pectiques. Dans ce chapitre, la structure des RGI et RGII, la production des Acs respectifs nécessaires pour la microscopie, ainsi que leur localisation *in planta* sont discutées.

L'étude de la structure des RGI et RGII reste difficile en dépit des progrès des méthodes analytiques. Les conditions d'extraction des polysaccharides, qui déterminent le rendement, peuvent aussi impacter la structure des polymères isolés. Malgré de nombreuses études, la caractérisation des domaines de RGI et RGII est, à ce jour, loin d'être complètement résolue. La détermination des structures est facilitée par la dégradation des polymères en petits fragments, plus faciles à analyser. Les hydrolyses enzymatiques et chimiques sont largement utilisées à cet effet. Néanmoins, le manque d'enzymes et la non-spécificité de la dégradation chimique empêchent parfois certains fragments d'être décrits de façon optimale.

Au cours de cette thèse, quatre immunisations ont été menées pour la génération d'anticorps (RUP, RUB, chaîne latérale A et chaîne latérale B). Certaines n'ont pas permis la sélection de clones intéressants (RUB, chaîne latérale A et chaîne latérale B). Deux des immunisations ont aussi été conduites en parallèle, à l'Université de Leeds (RUP, RUB). Des différences notables dans l'obtention des anticorps ont été identifiées par les deux institutions. Le type de glycane injecté a son importance car il est porteur ou non d'un

fragment immunogène. L'animal choisi peut également avoir une sensibilité différente à un type de motif glycosidique. Enfin, les méthodes utilisées pour le tri des clones peuvent privilégier la sélection de certains anticorps. En effet, ces méthodes peuvent être plus ou moins stringeantes, impliquant des sélections différentes d'anticorps. La caractérisation de l'épitope est un travail qui dépend incontournablement du type de glycane injecté dans l'animal.

La localisation des domaines pectiques dans la plante par immunocytochimie n'est parfois pas représentative de la réalité. Certains anticorps se fixent sur des structures proches, mais différentes, de celles décrites comme étant leur épitope. La complexité de la paroi cellulaire et l'abondance de certains composés peuvent parfois cacher certaines structures qui peuvent alors ne pas être accessibles par l'anticorps. La localisation hétérogène des domaines pectiques dans la paroi a été discutée.

Thesis outline

Pectin (from ancient Greek πηκτ ικός meaning coagulant) is an acidic polysaccharide present in plant cell walls. This heteropolymer is a prominent constituent of middle lamellae and primary cell walls in non-woody tissues, next to hemicelluloses and cellulose. Pectin is involved in a range of cell functions: it influences cell wall mechanical strength, porosity, pH, ion transport and cell-to-cell adhesion (Willats *et al.*, 2006) and, as a consequence, impacts plant growth and development. Furthermore, pectin-derived oligosaccharides have been shown to activate plant defense response and to stimulate morphogenesis (Caffall and Mohnen, 2009).

Pectin is widely used in food industry as a functional ingredient for its gelling, thickening and stabilizing properties (Sakai *et al.*, 1993; Willats *et al.*, 2006; Voragen *et al.*, 2009). The ability of pectin to increase viscosity and stabilize emulsions and suspensions is also used in pharmaceutical and cosmetic preparations (Sakai *et al.*, 1993; Madhav and Pushpalatha, 2002; Willats *et al.*, 2006). Today, pectin value has also been extended to other promising fields such as medicine, environment and energy. Indeed, pectin increasingly gains interest as possible positiveagent in animal and human health (Patel and Goyal, 2012; Leclere *et al.*, 2013). Its ability to chelate cations provides application in human and environmental detoxification (Eliaz *et al.*, 2006; Khotimchenko *et al.*, 2007; Krzesłowska *et al.*, 2009; Mata *et al.*, 2009; Leclere *et al.*, 2013). Finally, research in biofuel is another option that merits consideration for recycling the considerable amount of waste products from industrial processing of fruits and vegetables (Edwards and Doran-Peterson, 2012). Properties of

pectin *in planta* or as isolated polysaccharide and derived oligosaccharides depend highly on their structural features. Pectin is composed of different structural domains : homogalacturonan (HG), homogalacturonan analogues (rhamnogalacturonan II (RGII), xylogalacturonan, apiogalacturonan) and rhamnogalacturonan I (RGI) (Ridley *et al.*, 2001) Although the fine structure of these domains has been described to some extent, it is still unclear how they are linked to each other to form the whole pectin macromolecule. Furthermore, in plant tissues, according to developmental stages and plant sources, pectic molecules differ considerably. In the cell wall context, our knowledge of the pectin biosynthesis regulation and deposition is still incomplete. Furthermore, the way in which the different pectic domains contribute to cell wall biological and mechanical functions remains largely unknown.

The complex structure of pectin polymers has been studied by using a range of analytical tools including chromatographic, spectroscopic and immunolabelling techniques. However, to allow further progress in the understanding of the relationship between RGI structure and function *in planta*, novel pectin enzymes and probes are required. In the context of intact cell walls, a better definition of the pectic domains variability will lead to a better understanding of the structures' impact on biosynthesis and remodeling control.

My PhD project is part of a European Marie Curie Initial Training Network (ITN) project called WallTraC that has two major objectives:

- *To develop knowledge and new molecular tools & techniques for the analysis of plant cell walls & their component polymers - mainly pectin and cellulose - with direct applicability to industrial end-users in the functional food ingredients & plant fibre sectors;*

- *To provide high quality, intersectorial & trans-disciplinary training in plant cell wall analysis & complementary skills with the aim of enabling young scientists to respond to future demands in both academic and private sectors thereby securing the future EU expertise base.*

The aim of my thesis within the WallTraC project is to develop new antibodies (mAbs), which probe pectin structural motifs *in planta*. A set of mAbs specific to pectic structures is already

available. However, taken all together, they do not cover the whole polymer complexity. In particular, RGI complexity is poorly covered and there is no RGII mAb available.

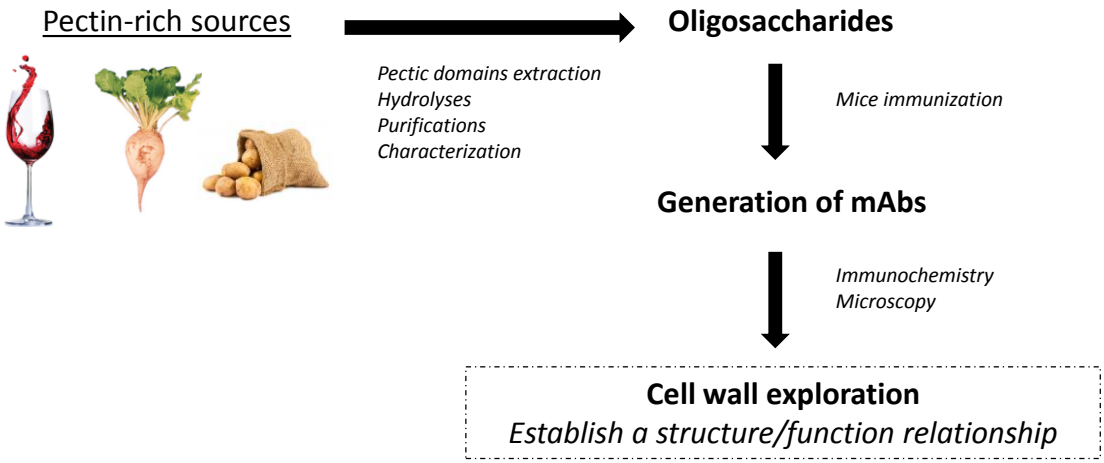


Figure D: Thesis main steps

The thesis researchwas organized in different steps (Figure.D), from pectin extraction to the use of produced mAbs to explore plant cell walls architecture. Oligosaccharides were produced from different plant sources using a combination of chemical and enzymatic tools and further purified, mainly by chromatographic means. RGI-related oligosaccharide pools were isolated from two different plant sources differing by their pectin side chain composition: sugar-beet root (arabinan-rich side chains) and potato tubers (galactan-rich side chains). RGII side chains A and B were obtained from wine RGII. The isolation of pectic oligosaccharides was performed in collaboration with two other PhD students within the WallTraC project: Valérie Cornault (University of Leeds, UK) produced the potato-derived RGI-related oligosaccharide pools and Jeff Zang (University of Newcastle, UK) purified whole RGII from wine. Both of them carried out this part of their work at INRA Nantes under the supervision of M.-C. Ralet. Pectic fragments were structurally characterized using different biochemical analytical methods and mass spectrometry. The oligosaccharides produced were then injected to mice in order to launch the immune system. During the thesis, 4 different immunizations were performed at INRA Nantes using the two pools of RGI-derived oligosaccharides and side chains A and B from RGII. Promising mAbs were obtained from potato RGI-derived oligosaccharides and were further characterized using glycan

microarrays. I carried out this part of my work in the laboratory of Pr. William Willats at the University of Copenhagen. All mAbs showed a new binding pattern on glycans and one of them was selected for additional studies to define the epitope. This mAb has also been tested in immunofluorescence microscopy on several plant species. Altogether, the results show that a new mAb against pectin RGI domain has been generated.

In the following document, an ***introduction*** chapter (chapter I) describes the state-of-the-art with respect to pectin structure and their functions *in planta*. Results on RGI and RGII are presented as two distinct chapters. ***Chapter II*** reports on the variation of structural features of wine rhamnogalacturonane II. ***Chapter III*** deals with the production and characterization of new monoclonal antibodies recognizing a specific RGI motif. A ***general discussion*** chapter (chapter IV) sums up the main results and exposes perspectives.

References

Caffall KH, Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate research* **344**(14): 1879-1900.

Edwards MC, Doran-Peterson J. 2012. Pectin-rich biomass as feedstock for fuel ethanol production. *Applied Microbiology and Biotechnology* **95**(3): 565-575.

Eliaz I, Hotchkiss AT, Fishman ML, Rode D. 2006. The effect of modified citrus pectin on urinary excretion of toxic elements. *Phytotherapy Research* **20**(10): 859-864.

Khotimchenko M, Kovalev V, Khotimchenko Y. 2007. Equilibrium studies of sorption of lead(II) ions by different pectin compounds. *Journal of Hazardous Materials* **149**(3): 693-699.

Krzesłowska M, Lenartowska M, Mellerowicz EJ, Samardakiewicz S, Woźny A. 2009. Pectinous cell wall thickenings formation : A response of moss protonemata cells to lead. *Environmental and Experimental Botany* **65**(1): 119-131.

Leclere L, Cutsem PV, Michiels C. 2013. Anti-cancer activities of pH- or heat-modified pectin. *Frontiers in Pharmacology* **4**: 128.

Madhav A, Pushpalatha PB. 2002. Quality upgradation of jellies prepared using pectin extracted from fruit wastes. *Journal of Tropical Agriculture* **40**(1/2): 31-34.

Mata YN, Blazquez ML, Ballester A, Gonzalez F, Munoz JA. 2009. Sugar-beet pulp pectin gels as biosorbent for heavy metals: Preparation and determination of biosorption and desorption characteristics. *Chemical Engineering Journal* **150**(2-3): 289-301.

Patel S, Goyal A. 2012. The current trends and future perspectives of prebiotics research: a review. *3 Biotech* **2**(2): 115-125.

Ridley BL, O'Neill MA, Mohnen DA. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**(6): 929-967.

Sakai T, Sakamoto T, Hallaert J, Vandamme EJ, Saul N, Allen IL. 1993. Pectin, pectinase, and Protopectinase: Production, Properties, and Applications. *Advances in Applied Microbiology* Academic Press, Volume 39: 213-294.

Voragen AGJ, Coenen G-J, Verhoef RP, Schols HA. 2009. Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry* **20**(2): 263-275.

Willats WGT, Knox JP, Mikkelsen JD. 2006. Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology* **17**(3): 97-104.

Achievement

Published article

- **Buffetto, F., Ropartz, D., Zhang, X. J., Gilbert, H. J., Guillon, F., Ralet, MC. 2014**. Recovery and fine structure variability of RGII sub-domains in wine (Vitis vinifera Merlot), *Annals of Botany*, **114**(6):1327-1337

Manuscript in preparation

- **Buffetto F., Cornuault V., Gro Rydahl M., Crépeau M.-J., Tranquet O., Willats W.G.T., Knox J.P., Guillon F., Ralet MC.** The deconstruction of pectic rhamnogalacturonan I unmasks the occurrence of a peculiar arabinogalactan epitope.

- **Cornuault V., Buffetto F., Gro Rydahl M., Marcus S., Torode T.A., Xue J., Crépeau M.-J., Willats W.G.T., Dupree P., Ralet M.-C., J. Knox P.** Monoclonal antibodies indicate low-abundance links between heteroxylan and other glycans of plant cell walls.

Oral communication

- **Buffetto F., Cornuault V., Gro Rydahl M., Crépeau M.-J., Tranquet O., Willats W.G.T., Knox J.P., Guillon F., Ralet M.C.** Production and fine characterization of new antibodies against Rhamnogalacturonan I. Journées du Réseau Français des Parois, Amiens, France, **07-09 July 2014.**

- **Buffetto F., Cornuault V., Gro Rydahl M., Crépeau M.-J., Tranquet O., Willats W.G.T., Knox J.P., Guillon F., Ralet M.C.** Production and fine characterization of new antibodies against Rhamnogalacturonan I. WallTraC Symposium, Paris, France, **17 May 2014.**

Poster

- **Buffetto F., Ropartz D., Zhang X.J., Gilbert H.J., Guillon F., Ralet M.C.** New insight into fine structure of red wine RGII. XIIIth International Cell Wall Meeting, Nantes, France, **7-12 July 2013.**

- **Buffetto F., Cornuault V., Gro Rydahl M., Crépeau M.-J., Tranquet O., Willats W.G.T., Knox J.P., Guillon F., Ralet M.C.** Production and fine characterisation of new monoclonal antibodies against rhamnogalacturonan I. XIIIth International Cell Wall Meeting, Nantes, France, **7-12 July 2013.**

- **Buffetto F., Cornuault V., Crépeau M.-J., Tranquet O., Knox P., Guillon F., Ralet M.C.** Production of low branched rhamnogalacturonan I oligosaccharides from sugar beet pulp. Plant and Seaweed Polysaccharides Symposium, Nantes, France, **17-20 July 2012.**

- **Buffetto F., Cornuault V., Crépeau M.-J., Tranquet O., Knox P., Guillon F., Ralet M.C.** Production of low branched rhamnogalacturonan I oligosaccharides from sugar beet pulp. **Summer Course Glycosciences,** Groningen, The Netherlands, **03-07 June 2012.**

Training

-INRA-Nantes (Nantes, France): Mass spectrometry & Standards for quality management systems, **21 to 23 May 2014**

-University of Newcastel (Newcaslte-upon-Tyne, United Kingdom): Cloning and characterization of recombinant proteins & Grant proposal writing, **20-22 November 2013**

-University of Postdam (Potsdam, Germany): Systems biology-oriented bioinformatics & EU project management, **13-17 June 2013**

-University of Leeds (Leeds, United Kingdom): Plant cell walls molecular probes & Ethics**, 14-16 January 2013**

-Bayer CropScience (Ghent, Belgium): Plant cell wall architecture & Commercial exploitation of results, **11-14 June 2012**

-CP Kelko, (Lille Skensved, Denmark): Introduction to industrial pectin production & Regulatory affairs, **16-18 November 2011**

CHAPTER I

State-of-the-art

Plant cells are surrounded by an elaborated extracellular matrix, the cell wall. The cell wall is a very dynamic entity, which gives to the cell rigidity and resistance against mechanical constraints. The whole sophisticated arrangement is predominantly constituted by polysaccharides (60-85% of the dry material), proteins (6-15%) and lignins (2-10%). Component proportions can vary with respect to both the plant source and the cell developmental stage. Furthermore, the cell wall can also react to various stimuli, such as pathogens.

Three main classes of polysaccharides compose plant cell wall: cellulose, hemicelluloses and pectins. Cell wall glycans structural versatility has been largely described and polysaccharide fine structure differs widely within the plant cell wall. So far, how cell wall polysaccharides structural variability relates to specific biological functions within the cell wall is still poorly understood. Plant cell wall generally comprises three different levels of organisation differing in their polysaccharides composition.

The middle lamella is situated between two cells, at the most extreme position of each one (Figure.I.1). It is mainly made of pectic components. It is shaped during mitosis and ensures the overall cohesion of the adjacent cells. Young cells are surrounded by a primary cell wall (Figure.I.1). It is synthesized during the cell expansion stage, stability and extensibility being

the mechanical properties required for cell growth. Primary cell wall is highly hydrophilic (60% of water). It is made of a loose network of cellulose microfibrils combined with hemicelluloses and pectins. When cell expansion is over and the cell does not need to grow anymore, the secondary cell wall can take place to thicken the extracellular matrix (Figure.I.1). Primary and secondary cell walls can be differentiated by their pectin and lignin content and by the structure of their hemicellulosic compounds. In secondary cell walls, the pectin content decreases dramatically and is mainly replaced by lignin. Cell wall loses its flexibility and becomes thicker to form a consolidated material.

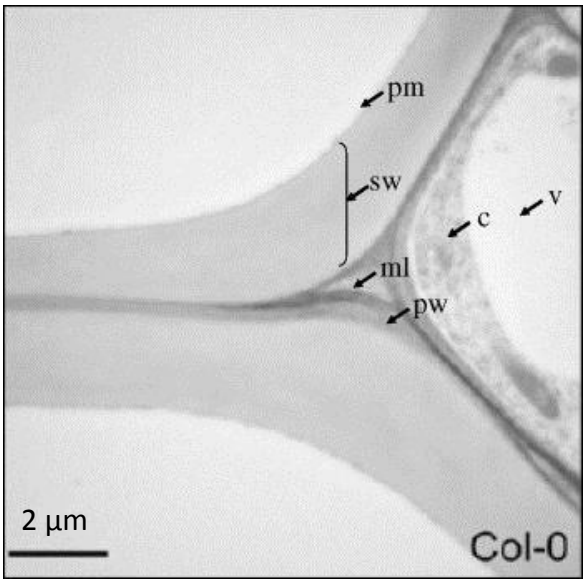


Figure I.1: The cell wall of *Arabidopsis thaliana* (wild type Columbia (Col-0)). From electron micrograph, all different entities of the wall are clearly recognizable in *Arabidopsis thaliana* metaxylem: middle lamella (ml), primary wall (pw) and secondary wall (sw). Plasma membrane (pm), cytosol (c) and vacuole (v) were also labeled. (From Caffall and Mohnen, 2009)

In this work, pectins were the focus of attention. To replace pectin in the context of primary cell walls in eudycotyledons, the other extra-cellular structures present in these cell walls are first briefly described. Pectin structure and the enzymes implicated in its biosynthesis and degradation are then reviewed. In the last part of this chapter, the biological role of the different pectic domains *in planta* is described.

I.1 Major components present with pectin in eudicotyledon primary cell walls

I.1.a Structural proteins

Proteins represent about 10 % of the cell wall mass (Tan *et al.*, 2013). Extra-cellular proteins can fulfil different functions in the wall. While some of them have a marginal role or still have unknown function, they can be organized under different functional classes. Proteins, which contain a catalytic site for reactions (i), are largely present in the apoplast. Enzymes are involved in various reactions (peroxidases, invertases, hydrolases, lyases, esterases), and have an active role in the extracellular matrix rearrangement or disassembling. Other proteins were described as having enzyme inhibitor roles (ii), such as the polygalacturonase inhibitor protein (PGIP) or the pectin methyl esterase inhibitor (PMEI). Some proteins are involved in cell signalling (iii) (Seifert and Roberts, 2007) or can operate as molecules transporters (iv) in the extracellular matrix. Finally, structural proteins (v) are required to maintain cell wall integrity.

Several hundreds of the structural proteins are present in the cell wall (Showalter, 1993). They can be rich in hydroxyproline (HRGP), proline (PRP) or glycine (GRP) amino acids. So far, the functional role of GRPs has not been clearly evidenced (Ringli *et al.*, 2001). HRGPs constitute the most studied group of structural proteins. They are classified in PRP, extensins and AGP. The three classes are characterized by repeated sequences of amino acids.

PRPs

PRPs represent one class of HRGPs, which are lowly glycosylated. PRPs are characterized by a sequence repetition based on a pentamer $(Pro\text{-}Hyp\text{-}Val\text{-}Tyr\text{-}Lys)_n$, which might also vary (Cassab, 1998). Transcription of PRPs is regulated over plant development (Wyatt *et al.*, 1992; Bernhardt and Tierney, 2000) and can also be induced by biotic and abiotic stresses (Sheng *et al.*, 1991).

Extensins

Extensins represent about 5% (dry material) of the primary cell wall in legumes and 0.5% in non-graminaceous plants. The monomeric molecule appears as a rod shape structure with an extended helical conformation (Lamport *et al.*, 2011). These glycoproteins contain a repeated sequence of $Ser(Hyp)_n$. Most of the Hyp are substituted by one to four α-L-Ara*f* units linked to each other by 1,2 or 1,3 linkages (Clarke *et al.*, 1979; Lamport *et al.*, 2011). The Ser residues can carry one or two β-D-Gal*p* units (Figure.I.2). Glycosylation is likely to maintain the helix stable and may also prevent extension enzymatic degradation.

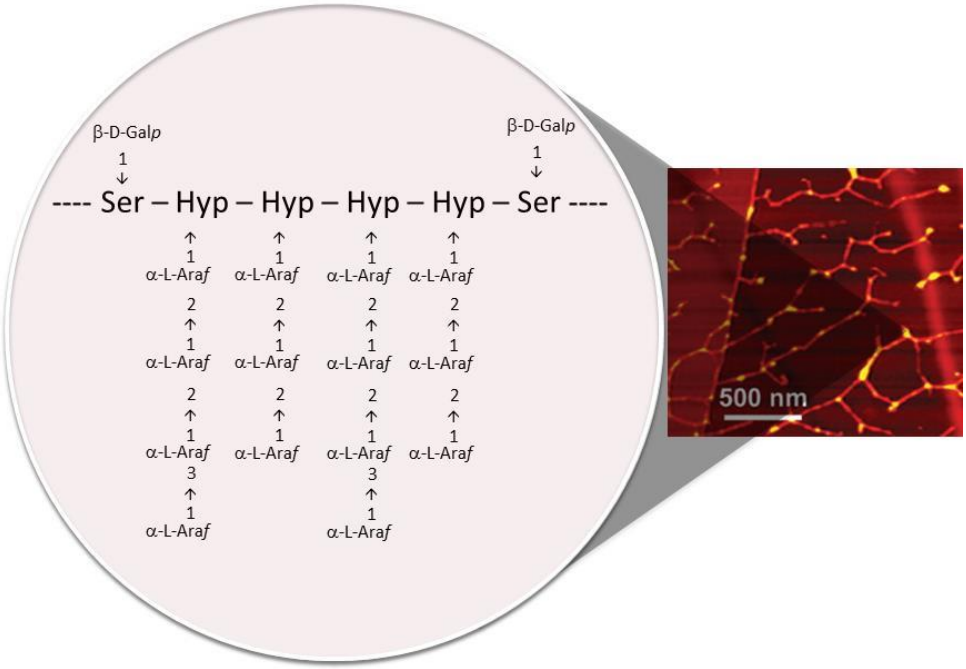


Figure I.2: Extensin self-assembly *via* hydrophobic segments (AFM image from Cannon *et al.*, 2008) and focus on the hydrophilic motif structure (Clarke *et al.*, 1979).

The glycosylated hydrophilic sequences (Figure.I.2) are interspersed with hydrophobic motifs, which are initiated by extensin peroxidase. Self-assembly builds a rigid and planar network (Figure.I.2), which increases the tensile strength of the primary cell wall (Cannon *et al.*, 2008). Extensins form a network for cell wall extensibility. The glycosylated proteins are involved in several physiological processes, such as morphogenesis (Wu *et al.*, 2001), response to mechanical stresses (Salva and Jamet, 2001) or plant injuries (Guzzardi *et al.*, 2004).

AGPs

AGPs are complex proteins, which account from 1 to 10% of the wall matrix. Some of the glycoproteins can be associated to a glycosylphosphatidylinositol anchor (GPI) at the hydrophobic C-Terminal extremity (Schultz *et al.*, 2000; Schultz *et al.*, 2002; Showalter *et al.*, 2010). The lipidic anchoring allows the incorporation of AGPs to the plasma membrane. The glycoproteins are composed of at least 90% of carbohydrate. Glycan chains of type II arabinogalactans (AGII) and short arabinans are branched to the protein backbone *via* Hyp residues. Two different models have been proposed to describe the macromolecular organization of the carbohydrates over the protein (Figure.I.3). The *Wattle-blossom model* is characterized by a global spherical structure and contains a GPI anchor. On this model, short side chains of arabino-oligosaccharides are present on contiguous Hyp residues. AGIIs are more likely localized onto non-contiguous Hyp residues, which are more abundant (Fincher *et al.*, 1983; Ellis *et al.*, 2010). This structure has been found in AGPs from carrot and tobacco (Cheung *et al.*, 1995). The *twisted hairy rope model* that applies to Arabic gums has a characteristic repeated sequence of proteins bearing short arabino-oligosaccharides and long strips of AGII (Qi *et al.*, 1991; Ellis *et al.*, 2010). More recently it was shown that pectin and hemicellulose can be covalently linked to AGP *via* an AGII domain (Tan *et al.*, 2013). AGII was found associated to Rha*p* and Glc*p*A units (Tan *et al.*, 2004).

AGP function is still unclear and their structural heterogeneity suggests that they could fulfil several functions (Ellis *et al.*, 2010; Showalter *et al.*, 2010). Several studies listed by Ellis *et al.* (2010), have pointed the significance of the AGP role in plant growth and development. They have also been described to have an impact on the cellular adhesion (Carpita and McCann, 2000).

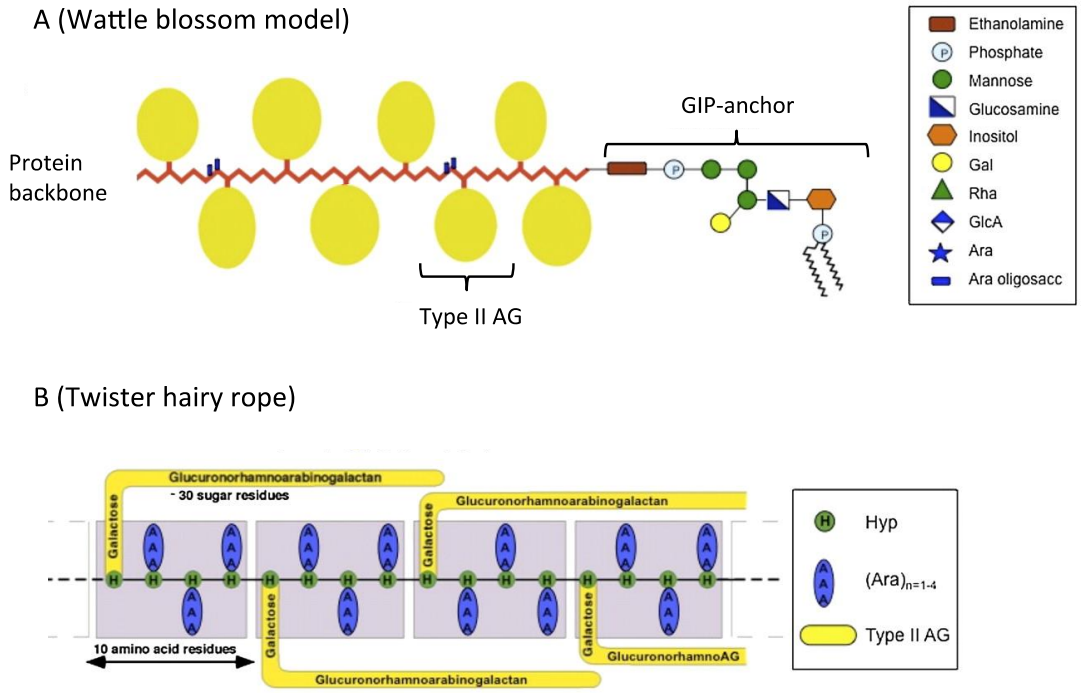


Figure I.3: Model of the glycan organization over the protein backbone of AGP (adapted from Ellis *et al.*, 2010). A: The wattle and blossom model shows the globular AGII (yellow ovals) and the short arabinan side chains (short blue dashes) beard by the protein backbone of the AGP. In this model, low amounts of contiguous Hyp residues are present. B: The twisted hairy rope model represents proteic sequences with contiguous Hyp, which are linked to short-arabino-oligosaccharides and elongated bands of glucuronorhamno-arabinogalactan.

I.1.b The cellulose and hemicellulose network

In contrast to the acidic pectin, cellulose and hemicelluloses are predominantly made of neutral sugars. Hemicelluloses are supposed to be associated to cellulose by non-covalent bonds forming the so-called cellulose-hemicellulose network.

Cellulose

Cellulose is the organic molecule the most present on Earth. It is insoluble and represents about 15 to 40% of the dry primary cell wall (Caffall and Mohnen, 2009). It consists of (1→4)-β-D-glucopyranoses, forming a linear homopolymer (Figure.I.4). Each chain may account for 2000 to 25000 residues.

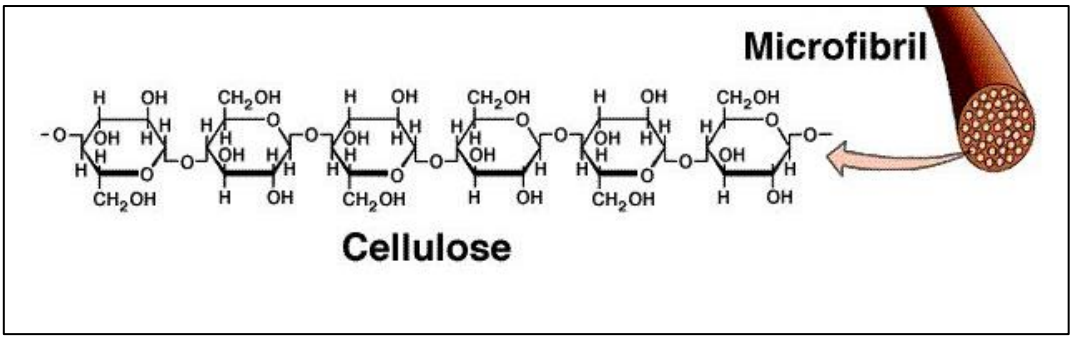


Figure I.4: Cellulose in the cell wall is arranged in microfibrils, which are paracristaline. Microfibrils are constituted of (1→4)-β-D-glucopyranoses chains bonded together by non-covalent linkages (Moore and Clark, 1998).

Cellulose is arranged to a special conformation by association of the polymeric glycan chains. Cellulose chains link through glycan interaction hydrogen bonding of free hydroxyls groups present on glucoses of the molecules. The hydrogen linkages enable to stabilize the linear parallel orientation of the cellulose molecule (Sugiyama *et al.*, 1991). Inter-chain connections result in a "multichain complex", which ensures a rigid construction. The whole structure drives to the generation of solid pseudo-crystalline fibers, which are organized together in microfibrils (Figure.I.4). This general conformation is solid and very condensed, which explains its insolubility in water. The center of the fiber has a crystalline structure whereas the cellulose chains located at the microfibrils periphery may have a less organized structure, (paracrystalline) (Earl and Vanderhart, 1981). Throughout the microfibrils, some alteration can occur and disrupt the condensate structure of the cellulose. The glucan chains are synthesized by terminal complexes or rosettes consisting of cellulose synthases (CESA) and associated enzymes. Present at the plasmic membranes, rosettes are formed by hexameric sub-units (Doblin *et al.*, 2002). Six CESAs are grouped to form a sub-unit. From UDP-D-Glc units, CESAs enable the production of 36 glucan chains, which are associated in a microfibril, giving a thickness of about 3-5nm (Somerville, 2006).

Hemicelluloses

Hemicelluloses represent about 25% of the primary cell wall dry material. They group all polysaccharides in the cell wall, which are neither cellulosic nor pectic. Hemicelluloses are usually extracted using dilute alkaline solutions. Hemicelluloses can have a homopolymeric

or heteropolymeric backbone. Homopolymeric backbones always have the same common structure constituted by (1→4)-β-D-glycopyranose units, which can be glucose, xylose or mannose (Figure.I.5). These hemicelluloses are called glucans, xylans or mannans, respectively. Heteropolymer backbones always involve glucopyranose residues, which can be linked to mannose residues (heteromannan) or linked to themselves with hetero-linkages (β(1-4)(1-3) glucans). Hemicelluloses may be substituted by various short side chains, which increase their structural diversity. Depending on the developmental stages or the plant species, different hemicellulosic polymers are present in the cell wall. In the primary cell wall of eudicotyledons, the most commonly encountered are xyloglucans, glucuronoarabinoxylans and glucomannans (Scheller and Ulvskov, 2010).

- *Xyloglucan*

Xyloglucan is the most abundant hemicellulosic polymer in eudicotyledon primary cell walls representing 20-25% of the dry material (Scheller and Ulvskov, 2010). Xyloglucan has a (1→4)-β-D-Glc*p* backbone that is branched at *O*-6 by α-D-Xyl*p* residues (Hayashi, 1989) (Figure.I.5A). Different xyloglucans have been described, based on the degree of substitution of the glucan backbone (Vincken *et al.*, 1997; Pauly *et al.*, 2013). The majority of higher plants produce xyloglucans, in which three of every four β-D-Glc*p* residues is substituted with a Xyl*p* residue. Less substituted xyloglucans containing only two Xyl*p* residues per four Glc*p* units have been identified in Solanales (Pauly *et al.*, 2013). Xyl*p* can be substituted at *O*-2 by β-D-Gal*p* or α-L-Ara*f* residues, depending on the plant source. Both substitutions can also coexist in the same plant (Hoffman *et al.*, 2005). β-D-Gal*p* residues, which are the most often observed, can be further substituted at *O*-2 by a α-L-Fuc*p* residue and/or acetylated at *O*-6 (Vincken *et al.*, 1997; Scheller and Ulvskov, 2010; Pauly *et al.*, 2013). Ara*f* unit can also be acetylated at *O*-6 and further substituted at *O*-3 by a β-L-Ara*f* residue (York *et al.*, 1996; Scheller and Ulvskov, 2010). Fry *et al.* (1993) have developed a nomenclature to represent xyloglucan ramifications. Only terminal sugars of the lateral chains are represented by a corresponding single letter, as represented in Figure.I.5A. The implication of xyloglucans in plant development has received much attention. Xyloglucans have a strong affinity to cellulose. They can be non-covalently linked to cellulose microfibrils *via* hydrogen bonds and may also be trapped in the cellulose structure, preventing the normal condensation of the microfibrils (Carpita and Gibeaut, 1993; Somerville, 2006; Anderson *et al.*, 2010). The

xyloglucan-cellulose interaction has long been thought to be crucial for primary cell wall architecture. The lack of detectable xyloglucan in the *xxt1 xxt2* double mutant resulted in significant changes in the mechanical properties of these plants (Cavalier *et al.*, 2008). Some xyloglucan oligomers were shown to induce cell expansion (McDougall and Fry, 1990). However, others may have a negative effect on cell expansion. For example, the nonasaccharide XXFG was demonstrated to inhibit pea stem growth (York *et al.*, 1984; McDougall and Fry, 1988). Xyloglucan can be also mobilized in cotyledons of seeds. The abundant accumulation of xyloglucan in these walls provides a carbohydrate source for the developing plant (Edwards *et al.*, 1985; dos Santos *et al.*, 2004).

- *Glucuronoarabinoxylan*

Glucuronoarabinoxylan is also present in primary cell walls of eudicotyledons where it represents approximately 5% of the dry material (Darvill *et al.*, 1980; Scheller and Ulvskov, 2010). Variability in xylan branching depends of the cell tissue and the development stage (Scheller and Ulvskov, 2010). In glucuronoarabinoxylans (Figure.I.5B), α-L-Ara*f* and α-D-Glc*p*A branching occurs at the *O*-2 position of a xylan backbone, which can also be acetylated at the *O*-2 and *O*-3 positions (Gille and Pauly, 2012). As the polysaccharide is mostly found in primary and secondary walls of grasses (Faik, 2010; Scheller and Ulvskov, 2010), potential functions in primary cell walls of eudicotyledons have been poorly studied. Also, the general role of xylans in the cell wall is not well defined. It has been pointed to play a role in cell wall thickening in eudicotyledon wood (Lee *et al.*, 2013). In the endosperm of cereal grains, glucuronoxylan was shown to act as a storage polymer (Naran *et al.*, 2009).

- *Glucomannan*

Glucomannan has a hetero-backbone of (1→4)-β-D-Man with interspersed β-D-Glc*p* residues (Pauly *et al.*, 2013) (Figure.I.5C). It represents 3-5% of the primary wall of eudicotyledons. The polymer can be acetylated at *O*-2 and *O*-3 on Man*p* residues. The functions *in planta* of this polymer remain a matter of debate. Mannan, which is considered to be the most ancient hemicellulose of the plant wall, is suggested to have been largely replaced by other hemicelluloses in spermaphytes (Scheller and Ulvskov, 2010; Pauly *et al.*, 2013) .

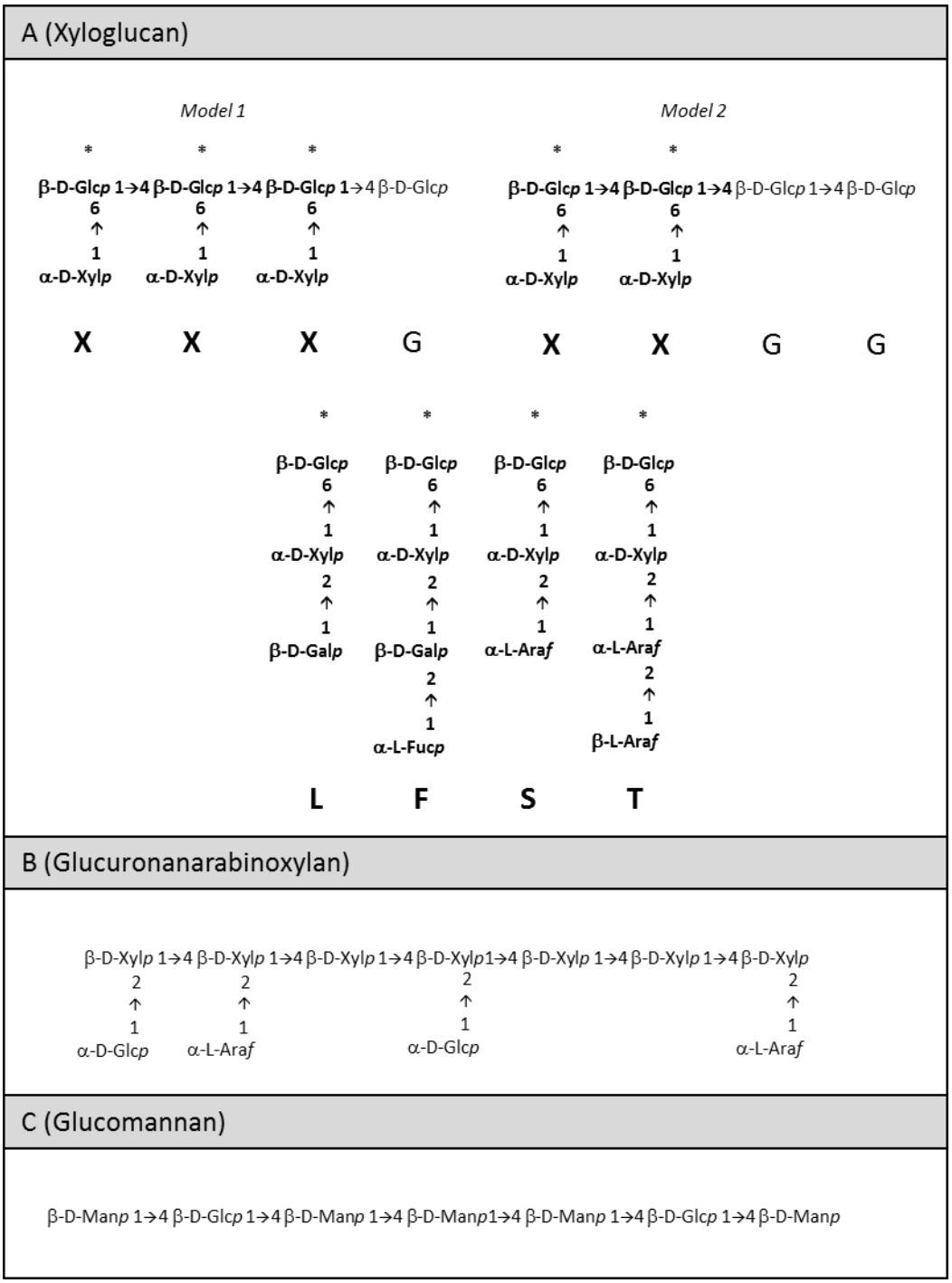


Figure I.5: Structure of the most encountered hemicelluloses in eudicotyledons primary cell walls. A: Xyloglucans. The two models of xyloglucan motifs are represented. In both models, short side chains, shown in blod (*), are variable. The β-D-Glc*p* unbranched residues in the backbone and the last sugar at the non-reducing end of each side chain are represented by a letter (G, X, L, F, S, T) (Fry *et al.*, 1993). B:Glucuronoarabinoxylan. C: Heterobackbone of glucomannan. All three polymers can be further acetylated at *O*-2 or *O*-3 on the glycosyl-pyranose from the backbone. Side chain of xyloglucan (A) can also be acetyled at *O*-6 on the β-D-Gal*p* or α-L-Ara*f* residues.

I.2 Pectins

Pectins are considered as key components for plant primary cell wall architecture in eudicotyledons and are involved in various cell functions and plant processes. They represent about one third of the primary cell wall. They are heterogeneous and complex galacturonic acid (Gal*p*A)-rich polysaccharides. These polymers are constituted by three major domains, which are covalently linked one to another (Caffall and Mohnen, 2009). The complex structure of homogalacturonans (HG), HG analogues and type I rhamnogalacturonans (RGI) requires a combination of many enzymes for their biosynthesis. HG and HG analogues are both constituted of a α-D-Gal*p*A backbone. In HG analogues (xylogalacturonan, apiogalacturonan, rhamnogalacturonan II), this backbone is branched by single sugar units or short side chains. RGI is the unique pectic domain having a heteropolymeric backbone made of alternating rhamnose (Rha*p*) and Gal*p*A residues. This heteropolymer can be substituted by various neutral sugar side chains. Whole pectin was shown to have a mechanical role and to be a strong adhesive material between cells (Carpita and Gibeaut, 1993; Willats *et al.*, 2001a; Jarvis *et al.*, 2003). Pectin is suggested to control porosity, pH, and hydration within the plant cell wall (Mohnen, 2008). Pectin-derived oligomers are deeply involved in cell signaling (Ridley *et al.*, 2001).

I.2.a Structure

Homogalacturonan

Homogalacturonan (HG) is the simplest and most abundant pectic structural domain, accounting for approximately 65% of the pectic domains. It consists of a linear backbone of (1→4) linked α-D-Gal*p*A residues (Figure.I.6A). The estimated length of this domain is 85-320 Gal*p*A residues (Thibault *et al.*, 1993; Hellin *et al.*, 2005; Yapo *et al.*, 2007; Round *et al.*, 2010). Gal*p*A residues are commonly partly methyl-esterified at *O*-6 (Voragen *et al.*, 1995) and, in some plant species, partly acetyl-esterified at *O*-2 or *O*-3 (Ralet *et al.*, 2005; Ralet *et al.*, 2008). Both the degree of methyl-esterification (DM) (i.e. the number of methyl-

esterified Gal*p*A residues for 100 total Gal*p*A residues) and degree of acetylation (DA) (i.e. the number of acetyl-esterified Gal*p*A residues for 100 total Ga*p*lA residues) have a profound impact on pectin gelling properties. HM pectins (≥ DM% 50) forms gels under acidic conditions in the presence of a cosolute whereas LM pectin (< DM% 50) forms gel by a different mechanism in the presence of calcium ions (May, 1990; Willats *et al.*, 2006). High DA has been shown to be a limiting factor for gelling properties (Vriesmann and Petkowicz, 2013). Not only the degree of the esterification but also the distribution of the esters is important. The distribution of esters is very complex at an intramolecular level (within one molecule) and at an intermolecular level (over various pectin molecules within a mixture) (Daas *et al.*, 1998; Daas *et al.*, 1999; Ralet *et al.*, 2008). The degree and distribution of esterification vary according to the plant species, age and location in the plant cell wall and is implicated in controlling functional properties of pectin (Bonnin *et al.*, 2008; Caffall and Mohnen, 2009).

Homogalacturonan analogues

- *Mono- or di-substituted homogalacturonans*

Mono- or di-substituted homogalacturonans (Figure.I.6B) can be branched by apiose or xylose units and/or disaccharide side chains (Le Goff *et al.*, 2001a; Le Goff *et al.*, 2001b). Apiogalacturonans, which are branched with a β-D-Api*f* at *O*-2 and/or *O*-3, were detected in the walls of aquatic plants (Hart and Kindel, 1970; Ovodov *et al.*, 1971; Ridley *et al.*, 2001). β-D-Api*f* disaccharides linked to the HG backbone were also isolated in duckweed. HG branched with β-D-Xyl*p* units at *O*-3 is called xylogalacturonan (Schols *et al.*, 1995; Le Goff *et al.*, 2001b; Zandleven *et al.*, 2006). Depending on plant sources, disaccharide side chains of β-D-Xyl*p* have been identified where *O*-2, *O*-3 and *O*-6 linkages were involved (Zandleven *et al.*, 2006). Concerning xylogalacturonan, a single evidence, for the methyl-esterification of the HG backbone, has been provided. In apple pectin, a purified fraction containing xylogalacturonan has been characterized in NMR spectroscopy to have a degree of methylation of 39 % (Schols *et al.*, 1995).

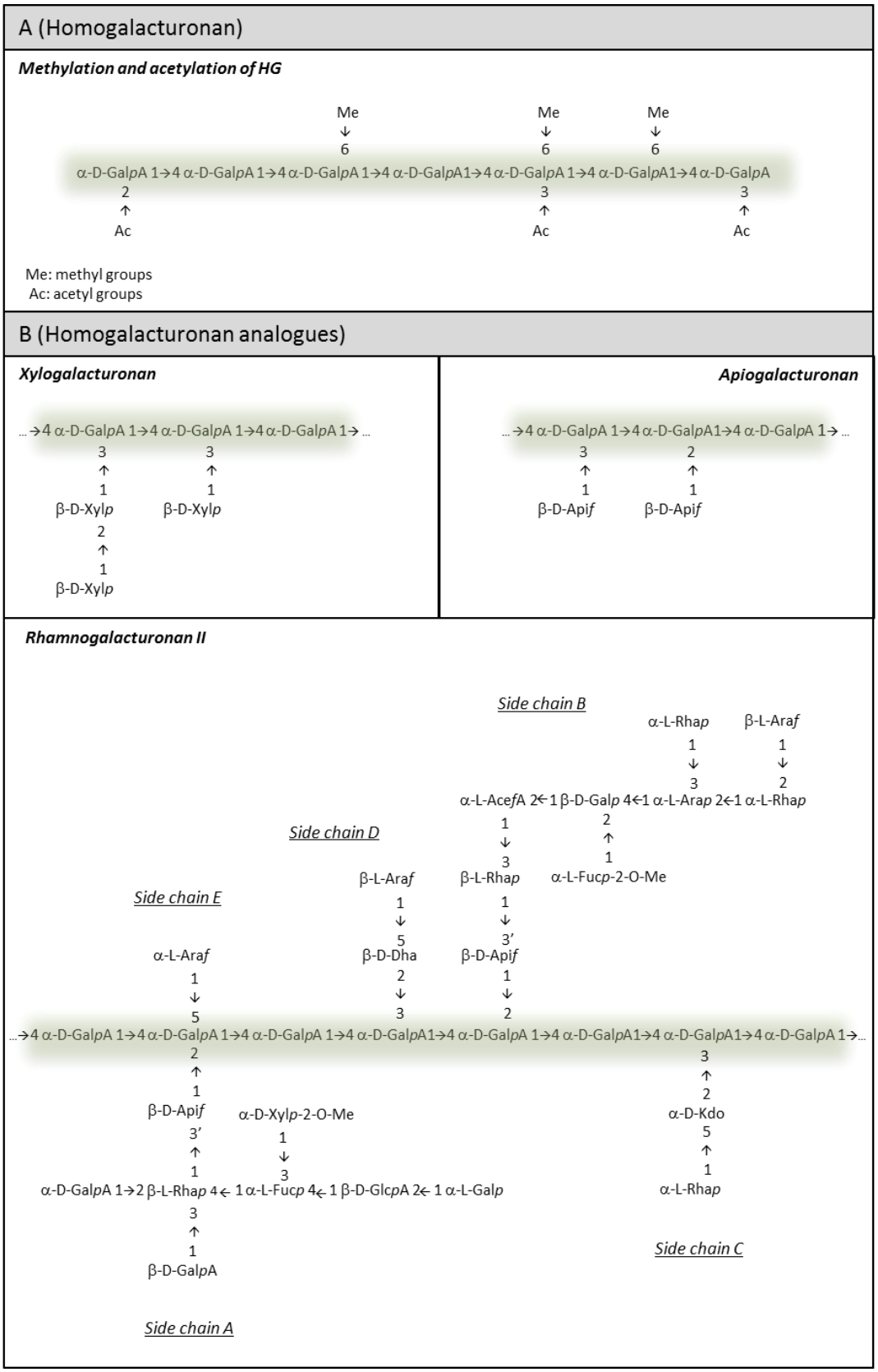


Figure I.6: Structural representation of HG and HG analogues. A: position of acetylation and methylation on Homogalacturonan. B: The three homogalacturonan analogues: xylogalacturonan; apiogalacturonan and rhamnogalacturonan II. In B, esterifications are not shown. However, only methylation has been well described in the RGII backbone.

Rhamnogalacturonan II is a low molecular weight (5-10kDa) highly complex macromolecule. It is indeed composed of 12 different glycosyl unit types and more than 20 different linkages (O'Neill *et al.*, 2004). It contains a backbone consisting of (1→4) linked α-D-Gal*p*A units substituted with 5 different side-chains (Figure.I.6B). The side chains include Gal*p*A, Rha*p*, Ara*f-p* and Gal*p* together with rare sugars such as aceric acid (Ace*f*A), methyl fucose (*O*-Me-Fuc*p*), methyl xylose (*O*-Me-Xyl*p*), 3-desoxy-D-*manno*-2-octulosonic acid (DHA), 3-desoxy-D-*lyxo*-2-heptulosonic acid (KDO). The whole structure of the RGII is extensively studied in Chapter II.

Rhamnogalacturonan I

- *Backbone*

RGI represents from 20% to 35% of the pectic polysaccharides (Mohnen, 2008). The RGI backbone is composed of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] repeats (Figure.I.7). This backbone was found to consist of up to 300 repeats (Schols *et al.*, 1990; Renard *et al.*, 1995; Albersheim *et al.*, 1996). Partial acetylation can occur at the *O*-2 and *O*-3 positions of the α-D-Gal*p*A residues (Komalavilas and Mort, 1989; Ishii, 1997) and less commonly at the *O*-3 of the α-L-Rha*p* residues (Komalavilas and Mort, 1989). No clear structural evidence of methylesterification of Gal*p*A carboxyl groups has been shown, but some RGI rich fractions were reported to contain limited amounts of methyl esters (Yapo, 2011).

- *Side chains*

RGI is decorated, mainly at the *O*-4 of the rhamnosyl units, by arabinan, galactan, arabinogalactan I and II side chains (Ridley *et al.*, 2001; Willats *et al.*, 2001a; Caffall and Mohnen, 2009; Yapo, 2011). From 20 to 80% of α-L-Rha*p* were reported to be substituted (Albersheim *et al.*, 1996; Ridley *et al.*, 2001). The proportion of side chains, their length and their degree of branching vary according to plant sources, taxons, organs and tissues (Lerouge *et al.*, 1993; Willats *et al.*, 2001a; Caffall and Mohnen, 2009). Arabinan-rich pectins have been isolated from apple, sugar beet, carrot and onion (Voragen *et al.*, 1995). Galactan

side chains are found in potato, soybean, and lupin (Voragen *et al.*, 1995). The RGI backbone Gal*p*A units can also be substituted by single β-D-Glc*p*A residues at *O*-3 (Renard *et al.*, 1999).

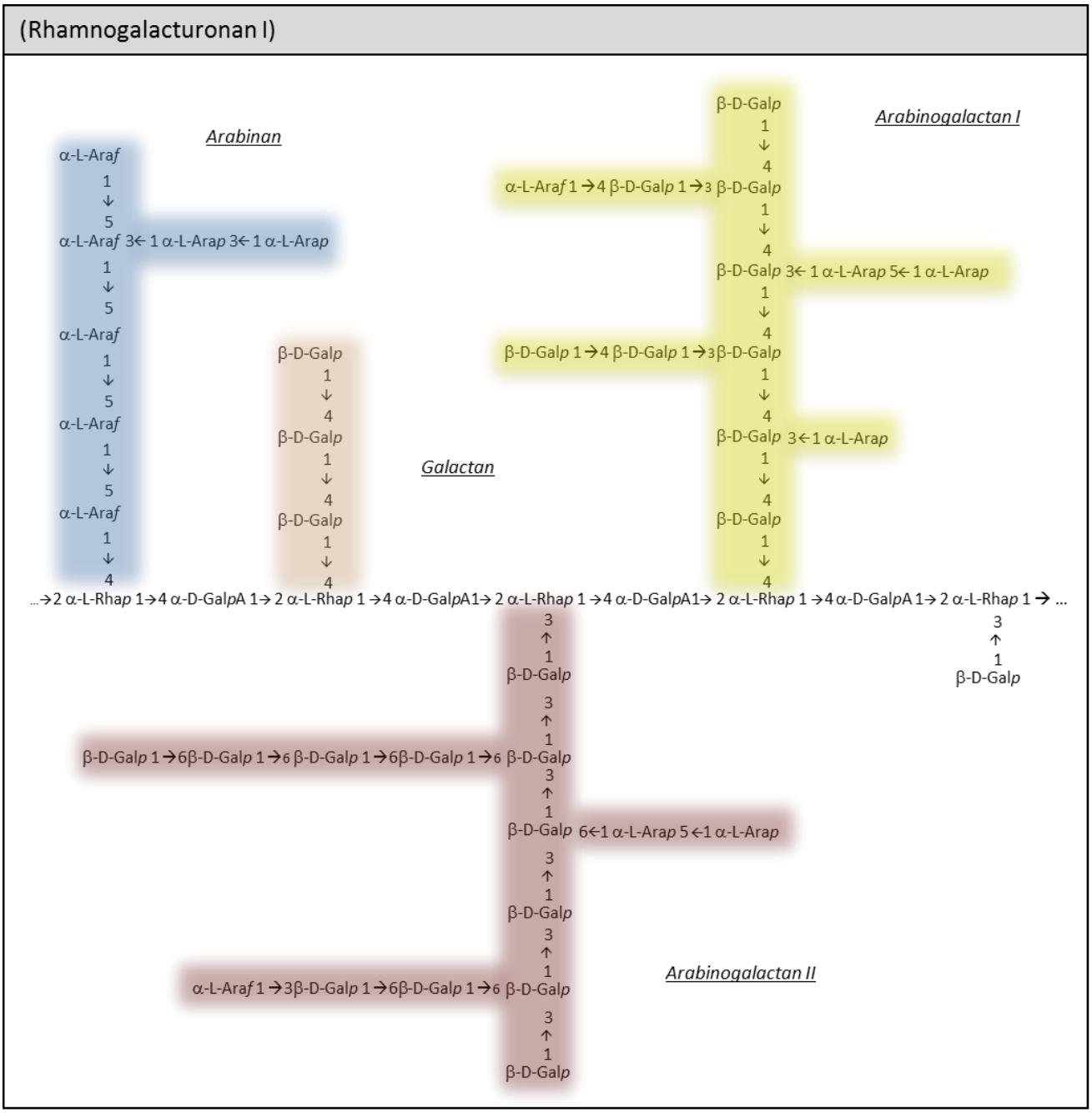


Figure I.7: Structural representation of Rhamnogalacturonan I. Different side chains may occur on this pectic domain. The length and proportion of arabinan, galactan, arabinogalactan type I and arabinogalactan type II side chains is plant- and tissue-specific.

L-α-Arabinans consist in a (1→5) linked α-L-Ara*f* backbone that can be substituted with (1→2) linked α-L-Ara*f* residues, (1→3) linked α-L-Ara*f* residues and/or by (1→3) linked α-L-

Ara*f* disaccharides (Carpita and Gibeaut, 1993; Ridley *et al.*, 2001). Galactan side chains encompass a (1→4)-β-D-Gal*p* backbone that may be branched at *O*-3 and *O*-6 by Gal*p* residues (Vis, 1994; hinz, 2005). Arabinogalactans are more complex molecules and consist of galactan chains bearing α-L-Ara*f* branches. Arabinogalactan I consists in a main (1→4)-β-D-galactan backbone, branched at *O*-3 with single α-L-Ara*f* unit or short (1→5)-α-L-Ara*f* chains (Carpita and Gibeaut, 1993; Ridley *et al.*, 2001). Complex Ara- and Gal-containing side chains were also evidenced in soybean (Huisman *et al.*, 2001). Indeed, oligosaccharides containing (1→4)-linked Gal*p* residues bearing one Ara*p* residue at the non-reducing end, and oligosaccharides made of (1→4)-β-D-Gal*p* residues interspersed with one internal (1→5)-α-L-Ara*f* residue were evidenced. Finally, (1→4)-β-D-Gal*p* residues interspersed with (1→3)-β-D-Gal*p* stretches were evidenced in potato, soybean, citrus and onion (Hinz *et al.*, 2005). Arabinogalactan II has a main (1→3)-β-D-galactan backbone, on which (1→6)-β-D-galactan secondary chains are branched (Carpita and Gibeaut, 1993; Ridley *et al.*, 2001; Mohnen, 2008). α-L-Ara*f* units can be attached at the *O*-3 position of the non-reducing end of the secondary galactan chains. Arabinogalactan II side chains non-reducing ends can be terminated by single α-L-Fuc*p*, β-D-Glc*p*A, 4-O-Me-β-D-Glc*p*A or β-D-Glc*p* residues (Ridley *et al.*, 2001; de Vries *et al.*, 2002).

Internal interaction of pectin structural domains

- *Glycosidic cross-linkages*

It is assumed that HG, HG analogues and RGI are covalently linked since harsh chemical and enzyme treatments are required to separate them one from another (Coenen, 2007). Different models have been proposed. In the “smooth and hairy region model” (Schols *et al.*, 1996), neutral sugars are concentrated in blocks of highly substituted RGI regions (designated hairy regions) separated by HG regions (designated smooth regions). In this model RGII is believed to be an integral part of some HG segments (Ishii and Matsunaga, 2001). In the “RGI backbone model” (Vincken *et al.*, 2003), HG is considered as a side chain of RGI attached to the rhamnosyl, or to Gal*p*A residues of the RGI backbone.

- *Hydrogen bonds*

Highly methylated homogalacturonans have the ability to self-aggregate *via* hydrogen bonds (Willats *et al.*, 2001a). Hydrogen bonds are thought to occur in the wall but this phenomenon has never been clearly observed.

- *Calcium-pectin complexes*

Calcium, a divalent cation, can act as an ionic bridge between negatively charged unmethylated Gal*p*A residues in the HG domain. *In vitro*, ionic-mediated gelation through cations is the classical mechanism of LM-pectin gelation (Zaidel and Meyer, 2012). Blocks of 7–14 methyl free α-D-Gal*p*A residues are required for stable association with calcium (Daas *et al.*, 2001). The mechanism of calcium binding to the ionized carboxyl groups of the HG chains has been studied for 40 years. The "eggs box" model, in which two HG chains are oriented in parallel, was first proposed by Grant et al, (1973). However, more recent conformational studies showed that this model cannot provide the best entropic contribution to the pectate dimer. It was instead suggested that the two HG chains could be anti-parallel (Braccini and Perez, 2001; Cabrera *et al.*, 2008). *In planta*, this type of interaction has been suggested to occur mostly in the middle lamellae.

- *Borate ester cross-linked pectin*

RGII exists in primary walls as a dimer, *via* covalently cross-linked boron. Borate diester is *O*-3 and *O*-2 linked to apiosyl residues from side chain A (Ishii *et al.*, 1999; Ishii and Ono, 1999). This complex is thought to contribute to the formation of a three-dimensional pectic network that is believed to have a role in cell wall properties (Ishii *et al.*, 2001). This RGII-boron complex stabilized by Ca^{2+} may account for 95% of the RGII in plant (O'Neill *et al.*, 2001). About 80% of the plant boron is involved in the RGII dimerization via di-ester bonds with Api*f* residues.

- *Ferulic esters*

Pectins originating from *Chenopodiaceae* (spinach and sugar-beet for example) contain ferulic acid residues in the arabinan and galactan side chains (Fry, 1982; Clausen *et al.*, 2004). In arabinans, feruloylation occurs at the *O*-2 and *O*-5 position of α-L-Ara*f* (Ralet *et al.*, 2005). On galactan side chains, ferulic acid was detected at the *O*-6 position (Ralet *et al.*, 2005). These phenolic residues are potential sites for crosslinking *via* oxidative coupling.

Ferulic acid dehydrodimers account between 10% to 29% of the total ferulates in sugar-beet pectin (Levigne *et al.*, 2004).

- *Interconnections with other polysaccharides*

Evidence for pectin interconnection with the other cell wall polymers is increasing. The existence of a possible covalent linkage between xyloglucan and pectin was first suggested by Thompson and Fry (2000) based on the co-elution of a fraction of alkali-extracted xyloglucan from suspension-cultured rose cells with high anionic pectin on anion exchange chromatography under high salt concentration. Treatments with either endo-galacturonase or galactanase or arabinanase converted appreciable amounts of this anionic xyloglucan to neutral material. The xyloglucan-pectin bonding was further investigated by Popper and Fry (2008). Using pulse labeled Arabidopsis cell culture with [^{3}H] arabinose and monitoring the radiobelling of the anionic xyloglucan-pectin complex, they showed that the xyloglucan-pectin bonds were formed intra-protoplasmically. The proportion of the wall-bound [^{3}H]xyloglucan that was anionic was found almost constant at ~50% for several days, showing that the xyloglucan–pectin bond was stable. They concluded that pectin serves as a primer, on which xyloglucan is synthesized. The complex is then secreted into the apoplast, where the xyloglucan–pectin bonds are stable and the pectic moiety aids wall-assembly.

A pectin-like structure linked to xylan oligosaccharides was reported in soluble polysaccharides extracted from soybean cotyledons (Nakamura *et al.*, 2002). Indeed, short chains of β-D-Xyl*p* (dp up to 7) attached at the *O*-3 position of the HG were detected. More recently, a proteoglycan consisting of pectin and arabinoxylan covalently linked to an arabinogalactan protein were isolated from the media of Arabidopsis suspension cell culture (Tan *et al.*, 2013). The arabinoxylan is covalently linked, either on the RGI backbone via the β-D-Xyl*p* residues or on arabinogalactan via the α-L-Ara*f* residues (Tan *et al.*, 2013).

Non covalent linkage of RGI neutral side chains to cellulose was also investigated *in vitro* (Zykwinska *et al.*, 2005). Unbranched arabinan and galactan showed a high affinity to cellulose (Zykwinska *et al.*, 2005; Zykwinska *et al.*, 2006). It was suggested that pectin *via* some side chains may tether cellulose microfibrils (Zykwinska *et al.*, 2007).

I.2.b Pectin synthesis and glycosyl transferases

In contrast to cellulose, hemicelluloses and pectin are synthetized in the Golgi apparatus (Delmer, 1999). Pectin biosynthesis begins with activated nucleotide sugars necessary for the polymer growing. These precursors are from recycled cell wall polysaccharides and from plant photosynthesis. Enzymes *via* the salvage pathway, catalyze the conversion of free sugars in two sequential reactions involving sugar kinases and UDP-sugar pyrophosphorylases referenced by Atmodjo *et al*. (2013). Those recycled nucleotides sugars and those synthetized by photosynthesis metabolism are then substrates for nucleotide sugar interconverting enzymes, which extent the sugar nucleotide panel (Bar-Peled and O'Neill, 2011; Atmodjo *et al.*, 2013). Most of the enzymes are soluble and located in the cytosol. Nucleotide sugars are then transferred to the Golgi lumen and used as precursors for the complex pectin synthesis promoted by gycosyl-, methyl- and acetyl-transferases. Pectin polysaccharides are then transferred to the apoplast where they can be organized in the extracellular matrix (Driouich *et al.*, 1993). Over plant life, cell wall modification occurs and polysaccharides such as pectins undergo a continuous remodeling promoted by the action of the glycosyl hydrolases and esterases.

GlycosylTransferase (GT) activities have been reported already 45 years ago but isolation of pure and active enzymes is still challenging (Harholt *et al.*, 2010; Atmodjo *et al.*, 2013). To build the complex pectic polymer, about 67 different transferases are required (Mohnen, 2008; Harholt *et al.*, 2010). Putative enzymes have been identified but so far, only seven GTs involved in pectin biosynthesis have been functionally characterized. GTs are all suggested to be type II transmembrane proteins. The catalytic reactions are optimal in the pH range from 5 to 7 - usually found in the Golgi - and require cofactors such as Mn^{2+} or Mg^{2+}. Up to now, two different models have been proposed for pectin synthesis (Atmodjo *et al.*, 2013). In the consecutive GT model, individual GTs consecutively add sugar residues to the nonreducing end of growing oligo- or polysaccharides. In the domain synthesis model, each pectic domain would be synthetized one by one. After elongation, the domains would be connected one to another by hypothetical oligosyl-transferases.

Homogalacturonan

GAUT1 (GAlactUronyl-Transferase) and QUA1 (QUAsimodo)/GAUT8 are both well-studied GTs described to be involved in HG synthesis. They belong to the same GAUT sub-family from the CAZy GT8 family.

GAUT1 was the first functionally described protein identified in pectin biosynthesis. When expressed in the mammalian HEK293 kidney cells, this protein has an HG:GalAT activity (Sterling *et al.*, 2006). Using a reciprocal co-immunoprecipitation approach, GAUT1 has been recently shown to be complexed with GAUT7 (Atmodjo *et al.*, 2011). Despite the 60% similarity between both proteins, GAUT7 did not have any activity expression in the mammalian HEK293 kidney cells (Sterling *et al.*, 2006). Interestingly, the group of Mohnen showed that the type II transmembrane protein GAUT1 has its N-terminal transmembrane-spanning domain cleaved in the Golgi and thus, requires the GAUT7 association to anchor the complex to the plasma membrane (Atmodjo *et al.*, 2011). In the same study, immune precipitation allowed the identification of 12 other proteins that may be also associated to the GAUT1:GAUT7 complex. In *Arabidopsis thaliana*, the GAUT (36-68% identity to GAUT1) and GAUT-Like (23-29% identity to GAUT1) families have 15 and 10 putative members, respectively (Sterling *et al.*, 2006).

QUA1/GAUT8 is also a putative protein highly suggested to be involved in the homogalacturonan backbone synthesis (Bouton *et al.*, 2002). *Qua1* mutants show dwarfism and reduced cell adhesion. Cell wall chemical analysis revealed no major changes in neutral sugar content whereas a clear reduced amount of the Gal*p*A content was observed compared to the wild type. An additional study highlighted, after measurement in stem microsomal membrane, that the 1,4-D-xylanase synthase activity was also reduced by about 40% (Orfila *et al.*, 2005). Xylan content in stems was slightly decreased compared to the wild type. These results suggest that QUA1 could also be involved in xylan synthesis.

Homogalacturonan homologues

Few studies have been focussing on the synthesis of HG analogues. These polymers are constituted of a HG backbone substituted by monomers and/or rather short side chains.

GAUT proteins could be involved in the backbone synthesis before other proteins add side chains decorations.

- *Xylogalacturonan*

Xylogalacturonan deficient 1 (XGD1) is a gene encoding for a xylosyl-transferase, which transfers UDP-Xyl*p* onto Gal*p*A residues of the HG backbone (Jensen *et al.*, 2008). In the mutant *xgd1*, a reduction of 25% xylose content was observed in *Arabidopsis thaliana*. Hence, linkage analyses showed reduction of Xyl*p*T and 3,4-Gal*p*A, which are xylogalacturonan specific. Interestingly the study provides the evidence that RGI seems to offset the Xyl deficiency. An increase of 40% in Gal content was found in *xgd1* mutants, which mostly concerns the 1,4-linkages. An increase of the substituted Rha*p* supports the increase in galactan side chain amounts. In the *xgd1* mutant no specific growth phenotypes were detectable. Moreover, immunochemistry could not differentiate mutants and control using the LM8 probe, which recognizes xylogalacturonan (Willats *et al.*, 2004). Therefore, the authors hypothesized that *Arabidopsis thaliana* contains at least two different types of XGA, questioning the linkages produced by XGD1. The study highly supports that XGD1 is involved in the 1,3–β-D-Xyl*p* linkages but it has to be confirmed by additional biochemical analysis.

- *Rhamnogalacturonan II*

At least 22 GT are supposed to be required for the RGII synthesis. The biosynthesis of the most structurally complex polysaccharide has not been much studied except for a protein group from the GT77 CAZy family. Four protein members, RGXT1 to RGXT4, encoding for 1,3-α-D-xylosyltransferases have been identified and were showed to transfer α-D-Xyl*p* from UDP-Xyl*p* to α-L-Fuc*p*. This glycosyl linkage is only present in the RGII side chain A. The *rgxt4* mutant, in which pollen tubes and roots growth are affected, is the only one showing a clear phenotype (Liu *et al.*, 2011). The changeless phenotype of the *rgxt1* and *rgxt2* mutants is suggested being caused by genotype redundancy (Egelund *et al.*, 2006; Harholt *et al.*, 2010). The *rgxt3* mutant is not available (Liu *et al.*, 2011).

In *Nicotinia plumbaginifolia*, The NpGUT1 gene, which is altered in the *nolac-H18* line, was proposed to encode a glucuronyltransferase involved in the transfer of the Glc*p*A residue onto RGII side chain A (Iwai *et al.*, 2002). However, studies on the *Arabidopsis thaliana*

orthologous gene IRX10/IRX10L, provided evidence that GUT1 is more likely to encode for a gene involved in xylan biosynthesis (Wu *et al.*, 2001; Brown *et al.*, 2009). Furthermore analyses by mass spectrometry showed no structural changes in RGII on the double mutant *irx10*/*irx10l* when compared to the wild type (Seveno *et al*., 2009).

Rhamnogalacturonan I

The complex RGI made of different sugars and linkages requires a large number of enzymes for its synthesis. Only enzymes required in side chain synthesis will be presented in the following paragraphs. The GALS1 GT, involved in galactan synthesis, is the only gene encoding for an RGI-related GT that has been functionally characterized. From the CAZy GT 47 family, two putative enzymes (ARAD1 and XGD1) have been described for arabinan biosynthesis. GAUT11, which belongs to the CAZy GT8 family, might have an impact on the regulation of RGI backbone macromolecular properties (Kong *et al.*, 2013).

In the GT 92 family, three genes GALS1, GALS2 and GALS3 were recently described to encode for a 1,4- β-D-galactosyl-transferase (Liwanag *et al.*, 2012). The three genes appeared to be almost ubiquitously expressed in Arabidopsis but have distinct expression pattern in plant organ development. The three mutants *gals1*, *gals2* and *gals3* did not exhibit any notable morphological phenotype but all showed a decrease in Gal content, the most important decrease being for *gals1* mutant. GAL1 was selected for further characterisation. Both, overexpression of GAL1 in Arabidopsis, and glycosyltransferase assay using GALS1 protein isolated from microsomal fraction, led the authors to conclude that GALS1 encodes for a (1,4)-β-galactan synthase involved in the elongation of galactan chains.

In *Arabidopsis thaliana*, ARAD1 is a putative arabinosyl-transferase that was shown to have an impact on the cell wall arabinan content (Harholt *et al.*, 2006). Furthermore, linkage analyses showed a strong reduction in (1→5)-α-L-Ara*f* in the mutant compared to the wild type, which highly suggests that ARAD1 is a RGI (1→5)-α-L-arabinosyl-transferase. Functional activity remained to be characterized. ARAD1 was demonstrated to form a complex with its close homolog ARAD2 held together by sulfuric bonds (Harholt *et al.*, 2012). Despite they are closely related (65% amino acid homology), function of ARAD1 and ARAD2

were not redundant, since ARAD2 overexpression didn't rescue the biochemical phenotype of the *arad1* mutant. Furthermore, in contrast with wild type and the *arad1* mutant, immunochemistry analyses using the LM13 mAb revealed long stretches of (1→5)-α-L-Arabinan in intact roots of Arabidopsis seedling in the *arad2* mutant. Such changes in arabinan type detection led the authors to hypothesize that ARAD2 protein may be involved in adding lateral chains to the (1→5) arabinan core, either directly or through modulating the activity of other arabinosyl-transferases. *arad1* and *arad2* single mutants and the double *arad1 x arad2* mutant didn't show physiological growth phenotype changes compared to the wild type.

Acetyl and methyl transferases

Methyl and acetyl esterification on pectin are highly suspected to be Golgi-localized (Goubet and Mohnen, 1999; Pauly and Scheller, 2000; Ibar and Orellana, 2007). No functional enzymes have been characterized so far. Nevertheless, several mutants have been generated and studied. Cotton Golgi-related 3 (CGR3) and QUASIMODO 2 and QUASIMODO 3 (QUA2 and QUA3) were characterized as putative methyl transferases (Mouille *et al.*, 2007; Held *et al.*, 2011; Miao *et al.*, 2011). Mutants all showed pectin methylesterification deficiency, but additional studies are required to confirm the biochemical activity of the proteins. The only putative acetyltransferases described were xyloglucan-specific (Gille and Pauly, 2012).

I.2.c Enzymes involved in pectin degradation

Degradation of pectin is controlled by different classes of enzymes: esterases, hydrolases and lyases (Bonnin *et al.*, 2014). Glycan esterases remove non-carbohydrate substituants such as methyl and acetyl esters. Hydrolases are enzymes which are able to degrade glycosidic linkages between two different sugars in presence of water. Polysaccharide lyases cleave the glycosidic linkage by catalysing a β-elimination reaction. Depending on their mode of action, enzymes are classified into exo- or endo-enzymes. Exo-enzymes cleave off monomers or dimers from the non reducing end of the molecule, whereas endo-enzymes

hydrolyse the polymer substrate randomly. So far, no pectin trans-glycanase or trans-glycosidase has been identified in the wall. However, it is maybe a matter of time as transglycosylation reactions are required to integrate new synthetised pectic domains into the cell wall.

HG degrading enzymes

HG is deesterified by pectin methyl- and acetyl-esterases (PMEs – EC 3.1.1.11 - and PAEs – EC 3.1.1.6 -, respectively). PMEs can act on homogalacturonan, either randomly or linearly (Pelloux *et al.*, 2007; Wolf *et al.*, 2009). When PMEs act linearly, they give rise to blocks of free carboxylic groups that could interact with Ca^{2+} and contribute to cell wall stiffening. The mode of PME action depends on the pH, on the initial degree of methylesterification pattern or on the plant origin. PAEs remove acetyl groups from pectin. Theses enzymes are required since acetyl groups impede pectin dimerisation and inhibit enzymatic degradation through steric hindrance. In general, PAEs show broad substrate specificity and will de-acetylate both HG and RG-I (Bonnin *et al.*, 2008).

HG is depolymerised by endo-polygalacturonases (endo-PGs – EC 3.2.1.15 -), exo-polygalacturonases (Exo-PGs – EC 3.1.1.67 -) or by pectin and pectate lyases (PLs – EC 3.2.2.10 -, PALs – EC 3.2.2.2 -, Figure.I.8). Endo-PG and exo-PG catalyze the hydrolysis of glycosidic bond between two adjacent (1→4)-α-D-Gal*p*A units (Voragen *et al.*, 2009; Bonnin *et al.*, 2014). Previous polygalacturonan demethylation by PME makes HG more susceptible to degradation by PG. PL and PAL depolymerize HG by β-elimination reactions, introducing a double bond on the newly formed non reducing Gal*p*A end (Voragen *et al.*, 2009; Bonnin *et al.*, 2014). PLs act on methylated HG whereas PALs are specific of none or moderately methylated HG. The catalysis by PALs requires Ca^{2+}.

In planta, PME and PG (both endo and exo-acting) are present. They have been extensively studied in relation with fruit ripening, organ abscission or growth tube pollen (Sitrit *et al.*, 1999; Futamura *et al.*, 2000; Martin-Rodriguez *et al.*, 2002; Prasanna *et al.*, 2003; Brummell *et al.*, 2004; Supapvanich and Tucker). PAE and PAL have also been reported *in plants* (Bordenave *et al.*, 1995; Taniguchi *et al.*, 1995; Medina-Escobar *et al.*, 1997; Chourasia *et al.*,

2006; Wang *et al.*, 2010). These enzymes are present in numerous isoforms. However, more work is required to better understand the respective roles of these different isoforms in plant development events.

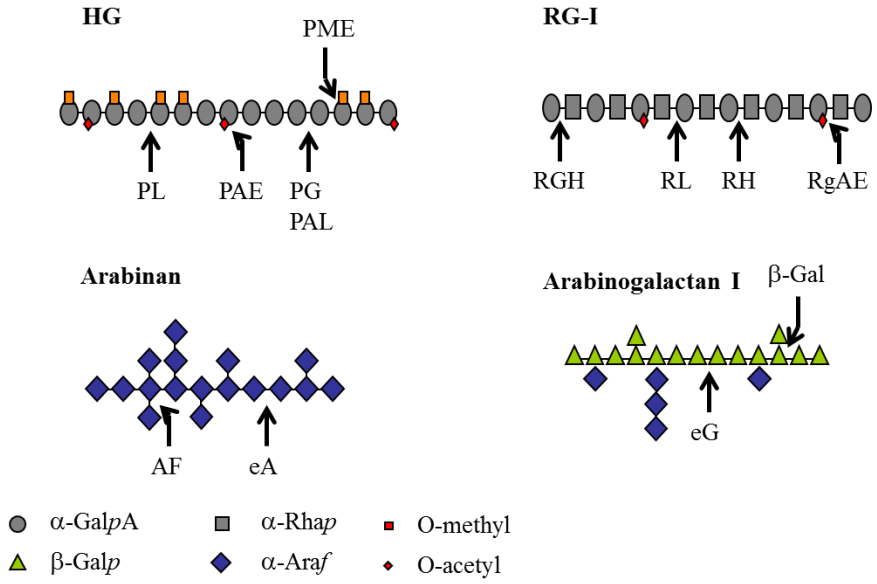


Figure I.8: Main pectin degrading enzymes (Bonnin *et al.*, 2014)

PG: polygalacturonase (endo, EC 3.2.1.15; exo, EC 3.2.1.67) ; PAL: pectate lyase (EC 4.2.2.2) ;
PL: pectin lyase (EC 4.2.2.10) ; PME: pectin methylesterase (EC 3.1.1.11) ;
PAE: pectin acetylesterase (EC 3.1.1.-); RH: rhamnogalacturonan hydrolase (EC 3.2.1.171) ;
RL: rhamnogalacturonan lyase (EC 4.2.2.23); RGH: rhamnogalacturonan galacturonohydrolase (EC 3.2.1.173);
eA: endoarabinanase (EC 3.2.1.99); AF: arabinofuranosidase (EC 3.2.1.55);
eG: endogalactanase (EC 3.2.1.89); β-Gal: β-galactosidase (EC 3.2.1.23).

RGI degrading enzymes

Acetyl groups on RGI are removed by acetyl esterase (RgAE - EC 3.1.1.86 -). RGI backbone is cleaved by RG hydrolases (main hydrolases: RH - EC 3.2.1.171- or RGH - EC 3.2.1.174-) and lyases (RL - EC 4.2.2.24 -) (Wong, 2008; Bonnin *et al.*, 2014). Arabinans side chains are degraded by endo-arabinosidases (eA – EC 3.2.1.99 -), exo-arabinanases (no Ec number), arabinofuranosidases (AF – EC 3.2.1.55 -). Galactasyl linkages are hydrolyzed in galactan and arabinogalactan type I by endo-galactanases (eG – EC 3.2.1.89 -), exo-galactanase (no Ec number), and β-galactosidases (β-gal – EC 3.2.1.23 -). The mode of action of these enzymes is described in Figure.I.8. These enzymes have been identified and extensively studied in different microorganisms, but only some of them are reported *in planta* (Wong, 2008; Goulao *et al.*, 2008; Bonnin *et al.*, 2014). For example, in the litterature, there is no mention

of endogenous endo-galactanases or endo-arabinanases. The presense of RG hydrolase in apple, grape and tomato fruit has been reported (Gross *et al.*, 1995). RG lyase activity has been found in cotton cotyledon intercellular spaces (Naran *et al.*, 2007). In fleshy fruit, β-galactosidase and α-arabinofuranosidase activities have been reported. Decline in the amount of galactose and arabinose from RGI side chains is a common feature in most ripening fruit. As a consequence, β-galactosidases and α-arabinofuranosidases are the RGI enzymes the most documented: some isoforms were described to have different expression pattern during fruit development and ripening with some present at high level only during ripening (Rose *et al.*, 2003; Brummell, 2006; Goulao *et al.*, 2008). Both β-galactosidase and exo-β-galactanase have been purified from lupin cotyledon but the exo-β-galactanase is the principal enzyme involved in galactan mobilization following germination (Buckeridge and Reid, 1994; Buckeridge *et al.*, 2005).

1.2.d Pectin distribution

To increase the understanding of the structure/function relationships of pectin *in planta*, the distribution of the structural domains has been investigated using immunocytochemical approaches (Willats *et al.*, 2001a). Several mAbs recognizing HG, RGI backbone, arabinan and (arabino)-galactan side-chains have been developed (Table.I.1). From an organism to another, in a given tissue, pectic composition may vary widely. However, specific occurrence of pectic domains has been highlighted. Young and meristematic cells are rich in methylesterified HG compared with elongating cells (Bush *et al.*, 2001; Leboeuf *et al.*, 2005; Sobry *et al.*, 2005). Cell development has also an impact on the RGI neutral side chains distribution. In different studies, arabinan rich side chains have been predominantly detected in proliferating cells (Willats *et al.*, 1999b) whereas the galactan rich side chains have been observed in the elongating cells and cambium (Willats *et al.*, 1999b; Ermel *et al.*, 2000; Bush *et al.*, 2001; McCartney *et al.*, 2003). Mucilage of Arabidopsis seed is mainly composed of unbranched RGI (Macquet *et al.*, 2007; Arsovski *et al.*, 2009). In peripheral layers of mature potato tuber (periderm) low methylated homogalacturonan has been identified while galactan and arabinan neutral side chains have been hardly detected (Bush and McCann, 1999; Sabba and Lulai, 2004). In contrast, RGI side chains are abundant in

potato tuber parenchyma or in cotyledon of leguminous seeds (Bush et al., 2001; Buckeridge et al., 2005; Habibi et al., 2005; Gomez et al., 2009).

Table I.1: Well characterised antibodies produced for pectin recognition

	antibodies	Epitope recognition	References
Homogalacturonan & HG analogues	2F4	HG unesterified Ca^{2+} cross-linked	-Liners *et al.*, 1989
	JIM5	HG partially methyl-esterified (DE=35<x<90%) and unesterified	-Knox *et al.*, 1990; Clausen *et al.*, 2003;Verhertbruggen *et al.*, 2009a
	JIM7	HG partially methyl-esterified (DE>50%)	-Knox *et al.*, 1990; Clausen *et al.*, 2003 Verhertbruggen, *et al*. 2009a
	PAM1*	HG unesterified long blocks	-Willats *et al.*, 1999a
	LM7	HG partially methyl-esterified	-Willats, *et al*., 2001b
	LM18	HG unesterified and partially methyl-esterified (preferred)	-Verhertbruggen *et al.*, 2009a
	LM19	HG unesterified (preferred) and methyl-esterified	-Verhertbruggen *et al.*, 2009a
	LM20	HG methyl-esterified	-Verhertbruggen *et al.*, 2009a
	LM8	Xylogalacturonan (but not all)	-Willats *et al.*, 2004
	Antisera**	Monomeric and dimeric RGII	-Matoh, 1998
Rhamnogalacturonan I	LM5	(1→4)-β-D-galactan	-Jones *et al.*, 1997
	LM6	Linear (1→5)-α-L-arabinan	-Willats *et al.*, 1998
	LM9	Feruloylated-(1→4)-β-D-galactan	-Clausen *et al.*, 2004
	LM13	Linear (1→5)-α-L-arabinan, binding can be increased by arabinofuranosidase pre-treatment	-Verhertbruggen *et al.*, 2009b
	LM16	(1→5)-α-L-arabinan. Epitope can be generated be arabinofuranosidase pre-treatment and is sensitive to β-galactosidase	-Verhertbruggen *et al.*, 2009b
	INRA-RU1	RGI backbone, it requires a minimum of six disaccharide repeats for the antibody binding (maximal binding for R_7U_7)	-Ralet *et al.*, 2010
	INRA-RU2	RGI Backbone, It requires at least two disaccharide (Rha*p*-Gal*p*A) repeats for the antibody binding (maximal binding from R_7U_7 to $R_9U_{9)}$	-Ralet *et al.*, 2010

All antibodies are monoclonal mAbs except for, PAM1* which has been generated from a naïve phage display library and the antisera from rabbit which contain polyclonal antibodies.**

The distribution of pectic domains has been investigated at the ultrastructural level using transmission electron microscopy. Esterified HG are detected throughout the cell wall whereas low methyl-esterified or de-esterified HG are often localized to middle lamellae, cell corners and around air spaces (Bush et al., 2001; Willats et al., 2001a; Leboeuf et al., 2005; Verhertbruggen et al., 2009a). In the wall, RGII is present in the primary cell walls but is absent from the middle lamellae (Matoh et al., 1998). Interestingly, linera (1→5)-arabinan and (1→4)-galactan are also localized in a different area of the wall. In mature potato tuber, (1→5)-arabinan is present throughout the cell wall, except at the extended middle lamella at cell corner (Bush et al., 2001; Oomen et al., 2002), whereas (1→4)-galactan is localized at the primary wall especially in the zone close to the plasmalemma and is absent from the middle lamella (Sørensen et al., 2000; Bush et al., 2001; Oomen et al., 2002). In seed tobacco endosperm, RGI backbone has been shown to have a distinct distribution pattern compared with side chains (Lee et al., 2013). RGI backbone is present in middle lamellae, and (1→5)-arabinan and (1→4)-galactan occur in adjacent primary cell wall regions.

I.2.e Pectin Functions

Currently, the relationship between pectin fine structure and function *in planta* is far from being clear. Pectin is a complex molecule mainly present in the primary cell walls and middle lamella of eudicot plants. This polyelectrolyte carbohydrate influences pH and ionic balance in the cell wall (Willats *et al.*, 2001a). Distribution of pectic domains is well controlled, and is continually rearranged by enzymes from the extracellular matrix. *In planta*, pectin has been shown to serve many functions playing a role in the mechanical and textural properties of tissues. The polymer is responsible for cell to cell adhesion and porosity of the wall which are both controlled by physico-chemical characteristics of pectin structure. However, in the context of cell wall, balance and roles of the individual pectic domains remain to be determined.

Homogalacturonan

- *HG contributes to cell adhesion and cell wall stiffening*

The capacity of HG to form gel is thought to be important for cell-to-cell adhesion and cell wall stiffening. This capacity is strongly influenced by the extent and the pattern of methyl esterification of HG. Calcium pectin complexes have been suggested to play a key role in cell adhesion. In particular, the Ca^{2+} cross-linked HG would contribute to the resistance to stress that tends to separate cells (Jarvis *et al.*, 2003). This hypothesis is supported by several studies. Low methyl esterified HG are found to occur at a consistent location within cell walls and intercellular matrices across a range of plant organs and species. Block of non methylesterified Gal*p*A (2F4 antibody epitope) when present is generally restricted to cell junction, middle lamella close to junction zones and the lining of intercellular spaces in plant tissues (Bush and McCann, 1999; Willats *et al.*, 1999b; Orfila *et al.*, 2001; Guillon *et al.*, 2008). In suspension-cultured carrot cells, a similar pattern distribution is observed (Liners and Vancutsem, 1992). In sugar beet compact calli, block of non methylesterified GalA is present in fibrillar material expanded in areas of cell separation, while it is not detected in friable callus (Liners *et al.*, 1994). The pectic *qua1* and *qua2* Arabidopsis mutants defective in a putative glycosyltransferase (Bouton *et al.*, 2002; Leboeuf *et al.*, 2005) and in a putative methyltransferase (Mouille *et al.*, 2007), respectively, show a reduced cell adhesion, a specific reduction in GalA and a lower labeling with antibodies recognizing blocks of non-methyl-esterified HG (2F4 and PAM1) observed in the middle lamellae at tricellular junction. Beside the localization of low methyl estrified HG, the distribution of calcium ions was investigated in some tissues by analytical ion microscopy (Roy *et al.*, 1994; Guglielmino *et al.*, 1997) and it was found that the cell corners were rich in Ca^{2+}. However, less than half of pectins are generally extracted by chelating agents and limited cell separation occurs in these conditions (Goldberg *et al.*, 1996), indicating that calcium mediated low methylated pectin cross-linking is not the only mechanism responsible for cell adhesion.

Proseus work's based on their results with the alga *Chara* corallina suggests that pectate chemistry may play a prominent role in the cell growth control. The wall extension is driven by turgor pressure that stretches the wall irreversibly as load-bearing bonds naturally break and re-form between wall polymers (Proseus and Boyer, 2006b). Calcium pectate bonds can

be distorted by tension from the turgor pressure, weakening the calcium bonding which holds the pectate complex together (Proseus and Boyer, 2006a). When the tension is above a threshold, the distortion would cause the calcium to be lost from the wall and bind to newly supplied pectate. The growth would be controlled when the new pectate forms new cross-links within the existing wall together with Ca^{2+} entering from the outside, thus strengthening the wall (Proseus and Boyer, 2007; Proseus and Boyer, 2008). Of course Chara has a simpler wall structure compared to land plants and the growth is not supported by wall polysaccharide cleavage enzymes. The non-enzymatic process of control growth has to be validated in land plants.

Reduced wall extensibility and cessation of growth in pollen tube and hypocotyls have been associated with a low level of pectin methylesterification (Bosch *et al.*, 2005; Parre and Geitmann, 2005; Derbyshire *et al.*, 2007). However, not all the PME mutants show a morphological growth phenotype and there is a discrepancy in the literature in the effect of demethylesterifiaction of HG on the tissue mechanical properties (Peaucelle *et al.*, 2013). Furthermore, the mechanism behind is not always easy to interpret as PME activity may have diverse consequences for wall properties, formation of calcium mediated cross-linking of HG, depolymerisation of HG by PG, release of Oligogalacturonides (OGA) with signaling function in the plant and change in the wall pH and ionic status.

Pectin solubilization and depolymerisation are among the most pronounced changes in the walls of ripening fruits and is generally correlated with changes with tissue softening (Brummell and Harpster, 2001; Rose *et al.*, 2003; Goulao *et al.*, 2008). In many ripening fruit PG and PME are abundant. In the ripening impaired tomato mutants, which exhibit delayed or reduced softening, the PG mRNA levels are low (Dellapenna *et al.*, 1987; Dellapenna *et al.*, 1989). These observations led to the hypothesis that PG mediated pectin depolymerization represented the enzymatic basis of softening. However, several attempts to control softening by genetic engineering of PME and PG involved in the pectin degradation during ripening had limited success (Rose *et al.*, 2003; Goulao *et al.*, 2008). These results stress that the full details of the mechanisms involved in fruit softening and by enlarge fruit texture are yet to be fully identified.

- *Oligogalacturonides related signaling*

When plant is attacked by pathogens (funghi, bacteria, virus), it produces phytoalexins, antimicrobial proteins and reactive oxygene species. Pectins are generally the first target of invading pathogens. Oligogalacturonides (OGA) are released by EPG or PL secreted from the pathogens. Those oligosaccharides interact with cell receptors. They influence the Ca^{2+} flux around the plasma membrane (Romani *et al.*, 2004) and induce signal transduction pathway for defense responses. OAG are also involved in the plant growth and development. OGA has been shown to inhibit auxin induced pea stem elongation (Branca *et al.*, 1988), to affect the expression of late auxin-responsive genes in tobacco (Mauro *et al.*, 2002). OGA has also been found to inhibit rhizogenesis (Bellincampi *et al.*, 1993), to induce the morphogenesis of flowering shoot growth (Marfa *et al.*, 1991), and to activate somatic mitosis (Altamura *et al.*, 1998). Except in fruit ripening, the OGA effect is generally the opposite of the effect of added auxin.

Rhamnogalacturonan II

RGII occurs in the primary cell walls of all higher plant (Matoh *et al.*, 1998). RGII can complex together with Boron, forming a borate–diol ester, which can crosslink two HG molecules. A boron deficiency affects RGII dimerisation and leads to a reduced plant growth that is accompanied by swelling of the cell walls, irregularly shaped cells (Matoh, 1997; Matoh *et al.*, 1998; Ishii *et al.*, 2001) and changes in the appearance of the middle lamella (Kouchi and Kumazawa, 1976; Ishii *et al.*, 2001). The tissues of boron-deficient plants are also often brittle. Supplying borate to the boron-deficient plants restores normal growth, reduces wall thickening, and increases the amount of RG-II cross-linking to normal levels (Ishii *et al.*, 2001). Additional evidence of the role boron RGII dimerization in plant growth has been provided with plants carrying the *bor1* mutation. These plants are extremely dwarfed (Noguchi *et al.*, 1997; Noguchi *et al.*, 2003). BOR1 is a gene encoding for a boron transporter protein. Plants carrying the *bor1* mutation cannot generate a sufficiently high concentration of borate in their xylem sap for transport to the shoot tissues to prevent boron deficiency from occurring. Growth is rescued with borate treatment (Noguchi *et al.*, 1997). The importance of cross linking RGII for normal plant growth has been also demonstrated with plant carrying the *mur1* mutation (O'Neill *et al.*, 2001). Alteration of the MUR1 gene

encoding for a guanosine diphosphate (GDP)–D-mannose-4,6-dehydratase changes the RGII sugar composition. α-L-Fuc*p* and 2-*O*-Me- α-L-Fuc*p* residues are replaced by α-L-Gal*p* and 2-*O*-Me- α-L-Gal*p.* The *mur1* plants are dwarfed and have brittle stems. The rate of dimer formation and stability of the cross link dimer in *mur 1* plants treated with exogenous α-L-Fuc*p* or borate are comparable to wild type and the growth is rescued (O'Neill *et al.*, 2001). Borate treatment also restores the tensile strength of *mur1* hypocotyls and inflorescence stems to near normal values. All together, these results suggest that boron RGII dimerisation has a fundamental structural role in the wall of land plants development.

Rhamnogalaturonan I

- *Pectic arabinan and galactan are mobile polymer components of hydrated cell walls in eudicot plants*

The contribution of each cell wall polymers in mechanical properties depends on the polymer's own rigidity and interaction with other polymers. Solid state magnetic resonance relaxation experiments have been used to study the rigidity and the spatial proximity of polysaccharides in cell walls of non gramineous plants. RGI side chains were found highly mobile within the hydrated cell wall. It was also shown that except in potato cell wall, arabinan side chains are more mobile than galactan one (Ha *et al.*, 2005; Larsen *et al.*, 2011; Ng *et al.*, 2013). This is in agreement with the stereochemistry of the (1,4)-β-D-galactopyranosyl and (1,5)-α-L-arabinofuranosyl linkages (Pérez *et al.*, 2000). In potato, (1→4) galactan chains are very long and a substantial fraction of each chain is far enough from the influence of RGI backbone attachment point to reorient continually (Ha *et al.*, 2005). It has been suggested that the side chains may contribute to the mechanical properties of the cell wall through physical entanglement. The high mobility of the arabinan and galactan segments makes it unlikely. Instead, their structural role could be as wall plasticizers and water binding agent during plant growth and development. However, according to Zykwinska *et al*., (2005, 2006, 2007) diverse pectic populations may coexist in the cell wall, one highly mobile and the other one displaying a restricted mobility and closely associated with cellulose surface. From Zykwinska's studies, it can be envisioned that

cellulose microfibrils can be tethered by some pectic side chains. Pectins together with xylogucans would control spacing between microfibrils cellulose and thus wall extensibility.

- *RGI arabinan is involved in wall plasticity*

The role of arabinans as plasticizer has been highlighted in several studies. Resurrection plants possess the ability to survive dehydration of their vegetative tissues for extended period of time and to recover a full metabolic competence upon rehydration. This leads to the question as to how these plants are able to withstand such substantial water loss and particularly the effect on the cell wall. Studies on the leave composition of these plants showed that the presence of arabinan and other Ara-rich polymers in high amount is a common feature (Moore *et al.*, 2008; Moore *et al.*, 2013). It has been hypothesized that these arabinan rich polymers acts as wall plasticizers ensuring the wall flexibility is maintained and rehydration is facilitated in these plants. By extension, it has been speculated that arabinans or galactans present in high amount in some seeds (Gomez *et al.*, 2009) are serving a dual function, protecting the seeds against desiccation and providing fuels to the growing embryo following germination.

The role of arabinans in wall flexibility has been also illustrated in the stomatal guard cells. The walls of guard cells undergo large and reversible deformation as a consequence of turgor pressure variation causing stomatal opening and closing (Jones *et al.*, 2003; Jones *et al.*, 2005). Treatment of cells with a combination of PME and PG caused stomata to open to a greater extent following fusicoccin induced opening in comparison to untreated cells whereas arabinanase or feruloyl esterase treatment impaired stomatal opening (Jones *et al.*, 2003; Jones *et al.*, 2005). Open stomata treated with arabinase and induced to close with either ABA or mannitol failed to close, suggesting that the walls were ‘locked’ into place. It is hypothesized that *in vivo*, the feruloylated RGI arabinans form ester linkages either to other feruloylated RGI arabinans or to other wall molecules and prevent the association of neighbouring regions of HG. It appears that pectin and phenolic esters have a conserved functional role in guard cells throughout contrasting angiosperm species.

Ulvskov *et al*., (2005) had investigated the significance of RGI structure for wall rheology, using potato tubers in which RGI structure has been remodeled *in vivo* using fungal enzymes. Tuber RGI molecules from the two transformed lines were reduced in linear galactans and branched arabinans, respectively. The transformed tuber tissues were found to be more brittle when subjected to uniaxial compression. The interpretation of the mechanical tests with the aid of mathematic model that takes into account the contribution of cellulose led the authors to propose that pectic matrix plays a role in transmitting stresses to the load bearing cellulose microfibrils and that small changes to the matrix environment impact the biophysical properties of the wall.

- *Unbranched RGI is involved in maintaining seed viability*

Seeds of a number of species, including the model plant Arabidopsis, release sticky mucilage on imbibition that is composed of complex polysaccharides. In Arabidopsis, unbranched RGI is the main component. This polymer is accumulated in the epidermal cells of the seed coat during their differentiation. Due to its hydrophilic nature, this biopolymer takes up water and expands on imbibition, rupturing the outer cell wall and forming a gelatinous halo that encapsulates the seed. It is generally suggested that seed mucilage aids water uptake into seeds. However, recent low-field NMR results showed that contrary to that belief, released mucilage trapped water and slowed its uptake into internal seed tissues (Saez-Aguayo *et al.*, 2014). By this way, it protects the seed against desiccation and prolongs the imbibed state, providing more time for DNA repair mechanisms to function and improve seed viability**.**

- *Arabinan in pectin is involved in cell attachement*

Several studies suggest that arabinan could be involved in cell to cell adhesion. The colorless non-ripening mutant (*cnr*) isolated from plantings of commercial tomato (*Lycopersicon esculentum*), grows similar to wild type fruits up to the mature-green stage, but does not ripen (Orfila *et al.*, 2001). In this mutant, large intercellular spaces in fruit pericarp were observed which indicates a lack of cell adhesion compared to the wild type. Immunolabeling with the LM6 antibody revealed the presence of arabinan in the cytosol suggesting that it is not incorporated into the walls at the pre-ripe stage. The mutant also has a reduced calcium binding capacity, possibly as consequence of suppressed HG de-esterification. These changes in cell walls of the *Cnr* tomato mutant with a reduced calcium complexed HG and a lack of

wall arabinan incorporation would suggest that these polymers may be implicated in cell adhesion. The role arabinan in intercellular attachment has also been proposed by Iwai *et al.*, (2001). T-DNA transformed *Nicotiana plumbaginifolia* non-organic callus (nolac-H14) with loosely attached constituent cells exhibits a drastic decrease in arabinose content. In particular the level of arabinan associated with hemicellulose fraction is reduced suggesting that arabinose-rich pectins, which are associated with hemicellulose-cellulose network, might play an important role in intercellular attachment (Iwai *et al.*, 2001).

- *Storage function of RGI long side chains in seeds*

Some species from dicots store galactans and arabinans in their seeds. These polysaccharides are degraded and metabolized as a source of carbon and energy for several metabolic processes in the embryo following germination (Crawshaw and Reid, 1984). Cotyledons of *Lupus angustilius* seed are rich in linear (1→4) linked galactan. It was estimated that galactan accounts for 70% of the total neutral sugars in the cell wall. The massive deposit of this polysaccharide occurs in the mesophyll of the cotyledon cell wall. After germination, 70% of the galactan have disappeared. An exo-galactanase has been demonstrated to be the key enzyme involved in the mobilization of the linear galactan (Buckeridge *et al.*, 2005). The remaining galactan may correspond to branched galactans resistant to the enzyme action (Buckeridge *et al.*, 2005). In this study, it was shown that other sugars are mobilized after germination but to a lower extent. In Brassicae seed cotyledon, arabinose is the main cell wall sugar (Qouta *et al.*, 1991; Gomez *et al.*, 2009). In *Arabidopsis thaliana* embryo, arabinose accounts for about 40% of the non cellulosic sugars (Gomez *et al.*, 2009). Histo-immunolabeling using the LM6 mAb reveals that the amount of (1→5) linked arabinan declines after germination. Experiments with C^{14}-labeled arabinose indicate that it is rapidly incorporated and metabolized in growing seedlings.

References

Albersheim P, Darvill AG, O'Neill MA, Schols HA, Voragen AGJ. 1996. An Hypothesis: The same six polysaccharides are components of the primary cell wall of all higher plants. In J. Visser, & A. G. J.Voragen. *Pectins and Pectinases* Amsterdam (Elsevier): 47-53.

Altamura MM, Zaghi D, Salvi G, De Lorenzo G, Bellincampi D. 1998. Oligogalacturonides stimulate pericycle cell wall thickening and cell divisions leading to stoma formation in tobacco leaf explants. *Planta* **204**(4): 429-436.

Anderson CT, Carroll A, Akhmetova L, Somerville C. 2010. Real-Time Imaging of Cellulose Reorientation during Cell Wall Expansion in Arabidopsis Roots. *Plant Physiology* **152**(2): 787-796.

Arsovski AA, Popma TM, Haughn GW, Carpita NC, McCann MC, Western TL. 2009. AtBXL1 Encodes a Bifunctional beta-D-Xylosidase/alpha-L-Arabinofuranosidase Required for Pectic Arabinan Modification in Arabidopsis Mucilage Secretory Cells. *Plant Physiology* **150**(3): 1219-1234.

Atmodjo MA, Sakuragi Y, Zhu X, Burrell AJ, Mohanty SS, Atwood JA, Orlando R, Scheller HV, Mohnen D. 2011. Galacturonosyltransferase (GAUT)1 and GAUT7 are the core of a plant cell wall pectin biosynthetic homogalacturonan: galacturonosyltransferase complex. *Proceedings of the National Academy of Sciences of the United States of America* **108**(50): 20225-20230.

Atmodjo MA, Hao Z, Mohnen D. 2013. Evolving views of pectin biosynthesis. *Annual Review of Plant Biology* **64**: 747-779.

Bar-Peled M, O'Neill MA. 2011. Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. *Annual Review of Plant Biology* **62**: 127-155.

Bellincampi D, Salvi G, De Lorenzo G, Cervone F, Marfà V, Eberhard S, Darvill A, Albersheim P. 1993. Oligogalacturonides inhibit the formation of roots on tobacco explants. *The Plant Journal* **4**(1): 207-213.

Bernhardt C, Tierney ML. 2000. Expression of AtPRP3, a proline-rich structural cell wall protein from arabidopsis, is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiology* **122**(3): 705-714.

Bonnin E, Clavurier K, Daniel S, Kauppinen S, Mikkelsen JDM, Thibault JF. 2008. Pectin acetylesterases from Aspergillus are able to deacetylate homogalacturonan as well as rhamnogalacturonan. *Carbohydrate Polymers* **74**(3): 411-418.

Bonnin E, Garnier C, Ralet MC. 2014. Pectin-modifying enzymes and pectin-derived materials: applications and impacts. *Applied Microbiology and Biotechnology* **98**(2): 519-532.

Bordenave M, Goldberg R, Huet JC, Pernollet JC. 1995. A novel protein from mung bean hypocotyl cell-walls with acetyl esterase-activity. *Phytochemistry* **38**(2): 315-319.

Bosch M, Cheung AY, Hepler PK. 2005. Pectin methylesterase, a regulator of pollen tube growth. *Plant Physiology* **138**(3): 1334-1346.

Bouton S, Leboeuf E, Mouille G, Leydecker MT, Talbotec J, Granier F, Lahaye M, Hofte H, Truong HN. 2002. Quasimodo1 encodes a putative membrane-bound glycosyltransferase required for normal pectin synthesis and cell adhesion in Arabidopsis. *Plant Cell* **14**(10): 2577-2590.

Braccini I, Perez S. 2001. Molecular basis of Ca2+-induced gelation in alginates and pectins: The egg-box model revisited. *Biomacromolecules* **2**(4): 1089-1096.

Branca C, Lorenzo GD, Cervone F. 1988. Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. *Physiologia Plantarum* **72**(3): 499-504.

Brown DM, Zhang Z, Stephens E, Dupree P, Turner SR. 2009. Characterization of IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in Arabidopsis. *Plant Journal* **57**(4): 732-746.

Brummell DA, Harpster MH. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Molecular Biology* **47**(1-2): 311-340.

Brummell DA, Dal Cin V, Crisosto CH, Labavitch JM. 2004. Cell wall metabolism during maturation, ripening and senescence of peach fruit. *Journal of Experimental Botany* **55**(405): 2029-2039.

Brummell DA. 2006. Primary cell wall metabolism during fruit ripening. *New Zealand Journal of Forestry Science* **36**(1): 99-111.

Buckeridge MS, Reid JSG. 1994. Purification and properties of a novel beta-galactosidase or exo-(1-->4)-beta-D-galactanase from the cotyledons of germinated lupinus-angustifolius l seeds. *Planta* **192**(4): 502-511.

Buckeridge MS, Hutcheon IS, Reid JS. 2005. The role of exo-(1-->4)-beta-galactanase in the mobilization of polysaccharides from the cotyledon cell walls of Lupinus angustifolius following germination. *Annals of Botany* **96**(3): 435-444.

Bush MS, McCann MC. 1999. Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiologia Plantarum* **107**(2): 201-213.

Bush MS, Marry M, Huxham IM, Jarvis MC, McCann MC. 2001. Developmental regulation of pectic epitopes during potato tuberisation. *Planta* **213**(6): 869-880.

Cabrera JC, Boland A, Messiaen J, Cambier P, Van Cutsem P. 2008. Egg box conformation of oligogalacturonides: the time-dependent stabilization of the elicitor-active conformation increases its biological activity. *Glycobiology* **18**(6): 473-82.

Caffall KH, Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate research* **344**(14): 1879-1900.

Cannon MC, Terneus K, Hall Q, Tan L, Wang YM, Wegenhart BL, Chen LW, Lamport DTA, Chen YN, Kieliszewski MJ. 2008. Self-assembly of the plant cell wall requires an extensin scaffold. *Proceedings of the National Academy of Sciences of the United States of America* **105**(6): 2226-2231.

Carpita NC, Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant journal for cell and molecular biology* **3**(1): 1-30.

Carpita NC, McCann M. 2000. The cell wall. *Biochemistry and Molecular Biology of Plants* American Society of Plant Physiologists, Rockville, MD: 52-109.

Cassab GI. 1998. Plant cell wall proteins *Annual Review of Plant Physiology and Plant Molecular Biology* **49**: 281-309.

Cavalier DM, Lerouxel O, Neumetzler L, Yamauchi K, Reinecke A, Freshour G, Zabotina OA, Hahn MG, Burgert I, Pauly M, Raikhel NV, Keegstra K. 2008. Disrupting two Arabidopsis thaliana xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *Plant Cell* **20**(6): 1519-1537.

Cheung AY, Wang H, Wu HM. 1995. A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell* **82**(3): 383-393.

Chourasia A, Sane VA, Nath P. 2006. Differential expression of pectate lyase during ethylene-induced postharvest softening of mango (Mangifera indica var. Dashehari). *Physiologia Plantarum* **128**(3): 546-555.

Clarke AE, Anderson RL, Stone BA. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry* **18**(4): 521-540.

Clausen MH, Willats WG, Knox JP. 2003. Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research* **338**(17): 1797-1800.

Clausen MH, Ralet M-C, Willats WGT, McCartney L, Marcus SE, Thibault J-F, Knox JP. 2004. A monoclonal antibody to feruloylated-(1→4)-ß-D-galactan. *Planta* **219**(6): 1036-1041.

Coenen GJ. 2007. *Structural characterization of native pectin*, Wageningen Universiteit.

Crawshaw LA, Reid JS. 1984. Changes in cell-wall polysaccharides in relation to seedling development and the mobilisation of reserves in the cotyledons of Lupinus angustifolius cv. Unicrop. *Planta* **160**(5): 449-454.

Daas PJH, Arisz PW, Schols HA, De Ruiter GA, Voragen AGJ. 1998. Analysis of Partially Methyl-Esterified Galacturonic Acid Oligomers by High-Performance Anion-Exchange Chromatography and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Analytical Biochemistry* **257**(2): 195-202.

Daas PJH, Meyer-Hansen K, Schols HA, De Ruiter GA, Voragen AGJ. 1999. Investigation of the non-esterified galacturonic acid distribution in pectin with endopolygalacturonase. *Carbohydrate Research* **318**(1-4): 135-145.

Daas PJH, Boxma B, Hopman AM, Voragen AG, Schols HA. 2001. Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers* **58**(1): 1-8.

Darvill JE, McNeil M, Darvill AG, Albersheim P. 1980. structure of plant-cell walls .11. glucuronoarabinoxylan, a 2nd hemicellulose in the primary-cell walls of suspension-cultured sycamore cells. *Plant Physiology* **66**(6): 1135-1139.

de Vries RP, Parenicova L, Hinz SWA, Kester HCM, Beldman G, Benen JAE, Visser J. 2002. The beta-1,4-endogalactanase A gene from Aspergillus niger is specifically induced on arabinose and galacturonic acid and plays an important role in the degradation of pectic hairy regions. *European Journal of Biochemistry* **269**(20): 4985-4993.

Dellapenna D, Kates DS, Bennett AB. 1987. Polygalacturonase gene-expression in rutgers, rin, nor, and nr tomato fruits. *Plant Physiology* **85**(2): 502-507.

Dellapenna D, Lincoln JE, Fischer RL, Bennett AB. 1989. Transcriptional analysis of polygalacturonase and other ripening associated genes in rutgers, rin, nor, and nr tomato fruit. *Plant Physiology* **90**(4): 1372-1377.

Delmer DP. 1999. Cellulose biosynthesis: Exciting times for a difficult field of study. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 245-276.

Derbyshire P, McCann MC, Roberts K. 2007. Restricted cell elongation in Arabidopsis hypocotyls is associated with a reduced average pectin esterification level. *Bmc Plant Biology* **7**.

Doblin MS, Kurek I, Jacob-Wilk D, Delmer DP. 2002. Cellulose biosynthesis in plants: from genes to rosettes. *Plant and cell physiology* **43**(12): 1407-1420.

dos Santos HP, Purgatto E, Mercier H, Buckeridge MS. 2004. The control of storage xyloglucan mobilization in cotyledons of Hymenaea courbaril. *Plant Physiology* **135**(1): 287-299.

Driouich A, Faye L, Staehelin LA. 1993. the plant golgi-apparatus - a factory for complex polysaccharides and glycoproteins. *Trends in Biochemical Sciences* **18**(6): 210-214.

Earl WL, Vanderhart DL. 1981. Observations by high-resolution c-13 nuclear magnetic-resonance of cellulose-i related to morphology and crystal-structure. *Macromolecules* **14**(3): 570-574.

Edwards M, Dea ICM, Bulpin PV, Reid JSG. 1985. Xyloglucan (amyloid) mobilization in the cotyledons of tropaeolum-majus l seeds following germination. *Planta* **163**(1): 133-140.

Egelund J, Petersen BL, Motawia MS, Damager I, Faik A, Olsen CE, Ishii T, Clausen H, Ulvskov P, Geshi N. 2006. Arabidopsis thaliana RGXT1 and RGXT2 encode Golgi-localized (1,3)-alpha-D-xylosyltransferases involved in the synthesis of pectic rhamnogalacturonan-II. *Plant Cell* **18**(10): 2593-2607.

Ellis M, Egelund J, Schultz CJ, Bacic A. 2010. Arabinogalactan-Proteins: Key Regulators at the Cell Surface? *Plant Physiology* **153**(2): 403-419.

Ermel FF, Follet-Gueye ML, Cibert C, Vian B, Morvan C, Catesson AM, Goldberg R. 2000. Differential localization of arabinan and galactan side chains of rhamnogalacturonan 1 in cambial derivatives. *Planta* **210**(5): 732-740.

Faik A. 2010. Xylan Biosynthesis: News from the Grass. *Plant Physiology* **153**(2): 396-402.

Fincher GB, Stone BA, Clarke AE. 1983. Arabinogalactan-proteins - structure, biosynthesis, and function. *Annual Review of Plant Physiology and Plant Molecular Biology* **34**: 47-70.

Fry SC. 1982. Phenolic components of the primary-cell wall - feruloylated disaccharides of d-galactose and l-arabinose from spinach polysaccharide. *Biochemical Journal* **203**(2): 493-504.

Fry SC, York WS, Albersheim P, Darvill A, Hayashi T, Joseleau JP, Kato Y, Lorences EP, Maclachlan GA, McNeil M, Mort AJ, Reid JSG, Seitz HU, Selvendran RR, Voragen AGJ, White AR. 1993. An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiologia Plantarum* **89**(1): 1-3.

Futamura N, Mori H, Kouchi H, Shinohara K. 2000. Male flower-specific expression of genes for polygalacturonase, pectin methylesterase and beta-1,3-glucanase in a dioecious willow (Salix gilgiana Seemen). *Plant and cell physiology* **41**(1): 16-26.

Gille S, Pauly M. 2012. O-acetylation of plant cell wall polysaccharides. *Frontiers in Plant Science* **3**.

Goldberg R, Morvan C, Jauneau A, Jarvis MC, Visser J, Voragen AGJ. 1996. Methyl-esterification, de-esterification and gelation of pectins in the primary cell wall. *Progress in Biotechnology* Elsevier, Volume 14: 151-172.

Gomez LD, Steele-King CG, Jones L, Foster JM, Vuttipongchaikij S, McQueen-Mason SJ. 2009. Arabinan metabolism during seed development and germination in Arabidopsis. *Molecular Plant* **2**(5): 966-976.

Goubet F, Mohnen D. 1999. Solubilization and partial characterization of homogalacturonan-methyltransferase from microsomal membranes of suspension-cultured tobacco cells. *Plant Physiology* **121**(1): 281-290.

Goulao LF, Cosgrove DJ, Oliveira CM. 2008. Cloning, characterisation and expression analyses of cDNA clones encoding cell wall-modifying enzymes isolated from ripe apples. *Postharvest Biology and Technology* **48**(1): 37-51.

Gross KC, Starrett DA, Chen H. 1995. Rhamnogalacturonase, alpha-galactosidase, and beta-galactosidase: potential roles in fruit softening.In: H. Hyodo and A. E. Watada. *Acta Horticulturae*: 121-130.

Guglielmino N, Liberman M, Catesson AM, Mareck A, Prat R, Mutaftschiev S, Goldberg R. 1997. Pectin methylesterases from poplar cambium and inner bark: Localization, properties and seasonal changes. *Planta* **202**(1): 70-75.

Guillon F, Philippe S, Bouchet B, Devaux M-F, Frasse P, Jones B, Bouzayen M, Lahaye M. 2008. Down-regulation of an Auxin Response Factor in the tomato induces modification of fine pectin structure and tissue architecture. *Journal of Experimental Botany* **59**(2): 273-288.

Guzzardi P, Genot G, Jamet E. 2004. The Nicotiana sylvestris extensin gene, Ext 1.2A, is expressed in the root transition zone and upon wounding. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1680**(2): 83-92.

Ha MA, Vietor RJ, Jardine GD, Apperley DC, Jarvis MC. 2005. Conformation and mobility of the arabinan and galactan side-chains of pectin. *Phytochemistry* **66**(15): 1817-1824.

Habibi Y, Mahrouz M, Vignon MR. 2005. Arabinan-rich polysaccharides isolated and characterized from the endosperm of the seed of Opuntia ficus-indica prickly pear fruits. *Carbohydrate Polymers* **60**(3): 319-329.

Harholt J, Jensen JK, Sorensen SO, Orfila C, Pauly M, Scheller HV. 2006. ARABINAN DEFICIENT 1 is a putative arabinosyltransferase involved in biosynthesis of Pectic Arabinan in Arabidopsis. *Plant Physiology* **140**(1): 49-58.

Harholt J, Suttangkakul A, Scheller HV. 2010. Biosynthesis of Pectin. *Plant Physiology* **153**(2): 384-395.

Harholt J, Jensen JK, Verhertbruggen Y, Sogaard C, Bernard S, Nafisi M, Poulsen CP, Geshi N, Sakuragi Y, Driouich A, Knox JP, Scheller HV. 2012. ARAD proteins associated with pectic Arabinan biosynthesis form complexes when transiently overexpressed in planta. *Planta* **236**(1): 115-128.

Hart DA, Kindel PK. 1970. Isolation and partial characterization of apiogalacturonans from the cell wall of Lemna minor. *The Biochemical journal* **116**(4): 569-579.

Hayashi T. 1989. Xyloglucans in the primary-cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 139-168.

Held MA, Be E, Zemelis S, Withers S, Wilkerson C, Brandizzi F. 2011. CGR3: A Golgi-Localized Protein Influencing Homogalacturonan Methylesterification. *Molecular Plant* **4**(5): 832-844.

Hellin P, Ralet MC, Bonnin E, Thibault JF. 2005. Homogalacturonans from lime pectins exhibit homogeneous charge density and molar mass distributions. *Carbohydrate Polymers* **60**(3): 307-317.

Hinz SW, Verhoef R, Schols HA, Vincken JP, Voragen AG. 2005. Type I arabinogalactan contains beta-D-Galp-(1-->3)-beta-D-Galp structural elements. *Carbohydrate Research* **340**(13): 2135-2143.

Hoffman M, Jia ZH, Pena MJ, Cash M, Harper A, Blackburn AR, Darvill A, York WS. 2005. Structural analysis of xyloglucans in the primary cell walls of plants in the subclass Asteridae. *Carbohydrate Research* **340**(11): 1826-1840.

Huisman MM, Brul LP, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AG. 2001. The occurrence of internal (1 --> 5)-linked arabinofuranose and arabinopyranose residues in arabinogalactan side chains from soybean pectic substances. *Carbohydrate Research* **330**(1): 103-114.

Ibar C, Orellana A. 2007. The import of S-Adenosylmethionine into the golgi apparatus is required for the methylation of homogalacturonan. *Plant Physiology* **145**(2): 504-512.

Ishii T. 1997. O-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiology* **113**(4): 1265-1272.

Ishii T, Matsunaga T, Pellerin P, O'Neill MA, Darvill A, Albersheim P. 1999. The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. *Journal of Biological Chemistry* **274**(19): 13098-13104.

Ishii T, Ono H. 1999. NMR spectroscopic analysis of the borate diol esters of methyl apiofuranosides. *Carbohydrate Research* **321**: 257-260.

Ishii T, Matsunaga T. 2001. Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry* **57**(6): 969-974.

Ishii T, Matsunaga T, Hayashi N. 2001. Formation of Rhamnogalacturonan II-Borate Dimer in Pectin Determines Cell Wall Thickness of Pumpkin Tissue. *Plant Physiology* **126**(4): 1698- 1705.

Iwai H, Ishii T, Satoh S. 2001. Absence of arabinan in the side chains of the pectic polysaccharides strongly associated with cell walls of Nicotiana plumbaginifolia non-organogenic callus with loosely attached constituent cells. *Planta* **213**(6): 907-915.

Iwai H, Masaoka N, Ishii T, Satoh S. 2002. A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem. *Proceedings of the National Academy of Sciences USA* **99**(25): 16319-16324.

Jarvis MC, Briggs SPH, Knox JP. 2003. Intercellular adhesion and cell separation in plants. *Plant Cell and Environment* **26**(7): 977-989.

Jensen JK, Sorensen SO, Harholt J, Geshi N, Sakuragi Y, Moller I, Zandleven J, Bernal AJ, Jensen NB, Sorensen C, Pauly M, Beldman G, Willats WGT, Scheller HV. 2008. Identification of a xylogalacturonan xylosyltransferase involved in pectin biosynthesis in Arabidopsis. *Plant Cell* **20**(5): 1289-1302.

Jones L, Seymour GB, Knox JP. 1997. Localization of Pectic Galactan in Tomato Cell Walls Using a Monoclonal Antibody Specific to (1-->4)-[beta]-D-Galactan. *Plant physiology* **113**(4): 1405-1412.

Jones L, Milne JL, Ashford D, McQueen-Mason SJ. 2003. Cell wall arabinan is essential for guard cell function. *National Academy of Sciences of the United States of America* **100**(20): 11783-11788.

Jones L, Milne JL, Ashford D, McCann MC, McQueen-Mason SJ. 2005. A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species. *Planta* **221**(2): 255-264.

Knox JP, Linstead PJ, King J, Cooper C, Roberts K. 1990. Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* **181**(4): 512-521.

Komalavilas P, Mort AJ. 1989. The acetylation at o-3 of galacturonic acid in the rhamnose-rich portion of pectins. *Carbohydrate Research* **189**: 261-272.

Kong Y, Zhou G, Abdeen AA, Schafhauser J, Richardson B, Atmodjo MA, Jung J, Wicker L, Mohnen D, Western T, Hahn MG. 2013. GALACTURONOSYLTRANSFERASE-LIKE5 is involved in the production of Arabidopsis seed coat mucilage. *Plant Physiol* **163**(3): 1203-1217.

Kouchi H, Kumazawa K. 1976. Anatomical responses of root tips to boron deficiency. III. Effect of boron deficiency on sub-cellular structure of root tips, particularly on

morphology of cell wall and its related organelles. *Soil Science and Plant Nutrition* **22**(1): 53-71.

Lamport DTA, Kieliszewski MJ, Chen YN, Cannon MC. 2011. Role of the Extensin Superfamily in Primary Cell Wall Architecture. *Plant Physiology* **156**(1): 11-19.

Larsen FH, Byg I, Damager I, Diaz J, Engelsen SB, Ulvskov P. 2011. Residue specific hydration of primary cell wall potato pectin identified by solid-state 13C single-pulse MAS and CP/MAS NMR spectroscopy. *Biomacromolecules* **12**(5): 1844-1850.

Le Goff A, Renard C, Bonnin E, Thibault JF. 2001a. Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls. *Carbohydrate Polymers* **45**(4): 325-334.

Le Goff A, Renard CMGC, Bonnin E, Thibault JF. 2001b. Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls. *Carbohydrate Polymers* **45**(4): 325-334.

Leboeuf E, Guillon F, Thoiron S, Lahaye M. 2005. Biochemical and immunohistochemical analysis of pectic polysaccharides in the cell walls of Arabidopsis mutant QUASIMODO 1 suspension-cultured cells: implications for cell adhesion. *Journal of Experimental Botany* **56**(422): 3171-3182.

Lee KJ, Cornuault V, Manfield IW, Ralet MC, Knox JP. 2013. Multi-scale spatial heterogeneity of pectic rhamnogalacturonan I (RG-I) structural features in tobacco seed endosperm cell walls. *Plant J* **75**(6): 1018-1027.

Lerouge P, O'Neill MA, Darvill AG, Albersheim P. 1993. Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydrate Research* **243**(2): 359-371.

Levigne SV, Ralet MC, Quemener BC, Pollet BN, Lapierre C, Thibault JF. 2004. Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiology* **134**(3): 1173-1180.

Liners F, Letesson JJ, Didembourg C, Van Cutsem P. 1989. Monoclonal Antibodies against Pectin: Recognition of a Conformation Induced by Calcium. *Plant Physiology* **91**(4): 1419-1424.

Liners F, Vancutsem P. 1992. Distribution of pectic polysaccharides throughout walls of suspension-cultured carrot cells - an immunocytochemical study. *Protoplasma* **170**(1-2): 10-21.

Liners F, Gaspar T, Vancutsem P. 1994. Acetyl-esterification and methyl-esterification of pectins of friable and compact sugar-beet calli - consequences for intercellular-adhesion. *Planta* **192**(4): 545-556.

Liu X-L, Liu L, Niu Q-K, Xia C, Yang K-Z, Li R, Chen L-Q, Zhang X-Q, Zhou Y, Ye D. 2011. MALE GAMETOPHYTE DEFECTIVE 4 encodes a rhamnogalacturonan II xylosyltransferase and is important for growth of pollen tubes and roots in Arabidopsis. *Plant Journal* **65**(4): 647-660.

Liwanag AJ, Ebert B, Verhertbruggen Y, Rennie EA, Rautengarten C, Oikawa A, Andersen MC, Clausen MH, Scheller HV. 2012. Pectin biosynthesis: GALS1 in Arabidopsis thaliana is a beta-1,4-galactan beta-1,4-galactosyltransferase. *Plant Cell* **24**(12): 5024-5036.

Macquet A, Ralet MC, Kronenberger J, Marion-Poll A, North HM. 2007. In situ, chemical and macromolecular study of the composition of Arabidopsis thaliana seed coat mucilage. *Plant Cell Physiol* **48**(7): 984-999.

Marfa V, Gollin DJ, Eberhard S, Mohnen D, Darvill A, Albersheim P. 1991. Oligogalacturonides are able to induce flowers to form on tobacco explants. *Plant Journal* **1**(2): 217-225.

Martin-Rodriguez M, Orchard J, Seymour G. 2002. Pectate lyases, cell wall degradation and fruit softening. *Journal of Experimental Botany* **53**(377): 2115-2119.

Matoh T, Takasaki M, Takabe K, Kobayashi M. 1998. Immunocytochemistry of Rhamnogalacturonan II in Cell Walls of Higher Plants. *Plant and cell physiology* **5**: 9.

Matoh T. 1997. Boron in plant cell walls. *Plant and Soil* **193**(1-2): 59-70.

Mauro ML, De Lorenzo G, Costantino P, Bellincampi D. 2002. Oligogalacturonides inhibit the induction of late but not of early auxin-responsive genes in tobacco. *Planta* **215**(3): 494-501.

May CD. 1990. Industrial pectins: Sources, production and applications. *Carbohydrate Polymers* **12**(1): 79-99.

McCartney L, Steele-King CG, Jordan E, Knox JP. 2003. Cell wall pectic (1→4)-β-d-galactan marks the acceleration of cell elongation in the Arabidopsis seedling root meristem. *The Plant Journal* **33**(3): 447-454.

McDougall GJ, Fry SC. 1988. Inhibition of auxin-stimulated growth of pea stem segments by a specific nonasaccharide of xyloglucan. *Planta* **175**(3): 412-416.

McDougall GJ, Fry SC. 1990. Xyloglucan oligosaccharides promote growth and activate cellulase - evidence for a role of cellulase in cell expansion. *Plant Physiology* **93**(3): 1042-1048.

Medina-Escobar N, Cardenas J, Moyano E, Caballero JL, Munoz-Blanco J. 1997. Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. *Plant Molecular Biology* **34**(6): 867-877.

Miao Y, Li HY, Shen J, Wang J, Jiang L. 2011. QUASIMODO 3 (QUA3) is a putative homogalacturonan methyltransferase regulating cell wall biosynthesis in Arabidopsis suspension-cultured cells. *Journal of Experimental Botany* **62**(14): 5063-5078.

Mohnen D. 2008. Pectin structure and biosynthesis. *Curr Opin Plant Biol* **11**(3): 266-77.

Moore JP, Farrant JM, Driouich A. 2008. A role for pectin-associated arabinans in maintaining the flexibility of the plant cell wall during water deficit stress. *Plant Signaling & Behavior* **3**(2): 102-104.

Moore JP, Nguema-Ona EE, Vicre-Gibouin M, Sorensen I, Willats WG, Driouich A, Farrant JM. 2013. Arabinose-rich polymers as an evolutionary strategy to plasticize resurrection plant cell walls against desiccation. *Planta* **237**(3): 739-754.

Moore R, Clark WD. 1998. Botany (3rd edition) McGraw-Hill.

Mouille G, Ralet MC, Cavelier C, Eland C, Effroy D, Hematy K, McCartney L, Truong HN, Gaudon V, Thibault JF, Marchant A, Hofte H. 2007. Homogalacturonan synthesis in Arabidopsis thaliana requires a Golgi-localized protein with a putative methyltransferase domain. *Plant Journal* **50**(4): 605-614.

Nakamura A, Furuta H, Maeda H, Takao T, Nagamatsu Y. 2002. Structural studies by stepwise enzymatic degradation of the main backbone of soybean soluble polysaccharides consisting of galacturonan and rhamnogalacturonan. *Bioscience, Biotechnology, and Biochemistry* **66**(6): 1301-1313.

Naran R, Pierce ML, Mort AJ. 2007. Detection and identification of rhamnogalacturonan lyase activity in intercellular spaces of expanding cotton cotyledons. *Plant Journal* **50**(1): 95-107.

Naran R, Black S, Decker SR, Azadi P. 2009. Extraction and characterization of native heteroxylans from delignified corn stover and aspen. *Cellulose* **16**(4): 661-675.

Ng JK, Schroder R, Sutherland PW, Hallett IC, Hall MI, Prakash R, Smith BG, Melton LD, Johnston JW. 2013. Cell wall structures leading to cultivar differences in softening rates develop early during apple (Malus x domestica) fruit growth. *BMC Plant Biology* **13**: 183.

Noguchi K, Yasumori M, Imai T, Naito S, Matsunaga T, Oda H, Hayashi H, Chino M, Fujiwara T. 1997. bor1-1, an Arabidopsis thaliana mutant that requires a high level of boron. *Plant Physiology* **115**(3): 901-906.

Noguchi K, Ishii T, Matsunaga T, Kakegawa K, Hayashi H, Fujiwara T. 2003. Biochemical properties of the cell wall in the Arabidopsis mutant bor1-1 in relation to boron nutrition. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **166**(2): 175-178.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG. 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* **294**(5543): 846-849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology* **55**: 109-139.

Oomen RJFJ, Doeswijk-Voragen CHL, Bush MS, Vincken J-P, Borkhardt B, Van Den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF. 2002. In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. *The Plant Journal* **30**(4): 403-413.

Orfila C, Seymour GB, Willats WGT, Huxham IM, Jarvis MC, Dover CJ, Thompson AJ, Knox JP. 2001. Altered middle lamella homogalacturonan and disrupted deposition of (1 -> 5)-alpha-L-arabinan in the pericarp of Cnr, a ripening mutant of tomato. *Plant Physiology* **126**(1): 210-221.

Orfila C, Sorensen SO, Harholt J, Geshi N, Crombie H, Truong HN, Reid JSG, Knox JP, Scheller HV. 2005. QUASIMODO1 is expressed in vascular tissue of Arabidopsis thaliana inflorescence stems, and affects homogalacturonan and xylan biosynthesis. *Planta* **222**(4): 613-622.

Ovodov YS, Ovodova RG, Bondarenko OD, Krasikova IN. 1971. The pectic substances of zosteraceae : Part IV. Pectinase digestion of zosterine. *Carbohydrate Research* **18**(2): 311-318.

Parre E, Geitmann A. 2005. Pectin and the role of the physical properties of the cell wall in pollen tube growth of Solanum chacoense. *Planta* **220**(4): 582-592.

Pauly M, Scheller HV. 2000. O-Acetylation of plant cell wall polysaccharides: identification and partial characterization of a rhamnogalacturonan O-acetyl-transferase from potato suspension-cultured cells. *Planta* **210**(4): 659-667.

Pauly M, Gille S, Liu L, Mansoori N, de Souza A, Schultink A, Xiong G. 2013. Hemicellulose biosynthesis. *Planta* **238**(4): 627-642.

Peaucelle A, Braybrook SA, Le Guillou L, Bron E, Kuhlemeier C, Hofte H. 2013. Pectin-induced changes in cell wall mechanics underlie organ initiation in Arabidopsis. *Current Biology* **21**(20): 1720-1726.

Pelloux J, Rusterucci C, Mellerowicz EJ. 2007. New insights into pectin methylesterase structure and function. *Trends in Plant Science* **12**(6): 267-277.

Pérez S, Mazeau K, Hervé du Penhoat C. 2000. The three-dimensional structures of the pectic polysaccharides. *Plant Physiology and Biochemistry* **38**(1-2): 37-55.

Prasanna V, Yashoda HM, Prabha TN, Tharanathan R. 2003. Pectic polysaccharides mango during ripening of (Mangifera indica L). *Journal of the Science of Food and Agriculture* **83**(11): 1182-1186.

Proseus TE, Boyer JS. 2006a. Calcium pectate chemistry controls growth rate of Chara corallina. *Journal of Experimental Botany* **57**(15): 3989-4002.

Proseus TE, Boyer JS. 2006b. Periplasm turgor pressure controls wall deposition and assembly in growing Chara corallina cells. *Annals of Botany* **98**(1): 93-105.

Proseus TE, Boyer JS. 2007. Tension required for pectate chemistry to control growth in Chara corallina. *Journal of Experimental Botany* **58**(15-16): 4283-4292.

Proseus TE, Boyer JS. 2008. Calcium pectate chemistry causes growth to be stored in Chara corallina: a test of the pectate cycle. *Plant Cell and Environment* **31**(8): 1147-1155.

Qi W, Fong C, Lamport DTA. 1991. Gum-arabic glycoprotein is a twisted hairy rope - a new model based on o-galactosylhydroxyproline as the polysaccharide attachment site. *Plant Physiology* **96**(3): 848-855.

Qouta LA, Waldron KW, Baydoun EAH, Brett CT. 1991. Changes in seed reserves and cell-wall composition of component organs during germination of cabbage (brassica-oleracea) seeds. *Journal of Plant Physiology* **138**(6): 700-707.

Ralet MC, André-Leroux G, Quéméner B, Thibault J-Fo. 2005. Sugar beet (Beta vulgaris) pectins are covalently cross-linked through diferulic bridges in the cell wall. *Phytochemistry* **66**(24): 2800-2814.

Ralet MC, Crepeau MJ, Bonnin E. 2008. Evidence for a blockwise distribution of acetyl groups onto homogalacturonans from a commercial sugar beet (Beta vulgaris) pectin. *Phytochemistry* **69**(9): 1903-1909.

Ralet MC, Tranquet O, Poulain D, Moise A, Guillon F. 2010. Monoclonal antibodies to rhamnogalacturonan I backbone. *Planta* **231**(6): 1373-1383.

Renard CM, Thibault JF, Mutter M, Schols HA, Voragen AG. 1995. Some preliminary results on the action of rhamnogalacturonase on rhamnogalacturonan oligosaccharides from beet pulp. *International Journal of Biological Macromolecules* **17**(6): 333-336.

Renard CM, Crepeau MJ, Thibault JF. 1999. Glucuronic acid directly linked to galacturonic acid in the rhamnogalacturonan backbone of beet pectins. *European Journal of Biochemistry* **266**(2): 566-574.

Ridley BL, O'Neill MA, Mohnen DA. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**(6): 929-967.

Ringli C, Keller B, Ryser U. 2001. Glycine-rich proteins as structural components of plant cell walls. *Cellular and Molecular Life Sciences* **58**(10): 1430-1441.

Romani G, Bonza MC, Filippini I, Cerana M, Beffagna N, De Michelis MI. 2004. Involvement of the plasma membrane Ca^{2+}-ATPase in the short-term response of Arabidopsis thaliana cultured cells to oligogalacturonides. *Plant biology (Stuttgart, Germany : Online)* **6**(2): 192-200.

Rose J, Catalá C, Gonzalez-Carranza C, H Z, J R. 2003. Plant cell wall disassembly.In: e. Rose JKC. *The plant cell wall* Oxford: Blackwell Publishing: 264-324.

Round AN, Rigby NM, MacDougall AJ, Morris VJ. 2010. A new view of pectin structure revealed by acid hydrolysis and atomic force microscopy. *Carbohydrate Research* **345**(4): 487-497.

Roy S, Jauneau A, Vian B. 1994. Analytical detection of calcium-ions and immunocytochemical visualization of homogalacturonic sequences in the ripe cherry tomato. *Plant Physiology and Biochemistry* **32**(5): 633-640.

Sabba RP, Lulai EC. 2004. Immunocytological comparison native and wound periderm maturation in potato tuber. *American Journal of Potato Research* **81**(2): 119-124.

Saez-Aguayo S, Rondeau-Mouro C, Macquet A, Kronholm I, Ralet M-C, Berger A, Salle C, Poulain D, Granier F, Botran L, Loudet O, de Meaux J, Marion-Poll A, North HM. 2014. Local evolution of seed flotation in Arabidopsis. *PLoS genetics* **10**(3): e1004221.

Salva I, Jamet E. 2001. Expression of the tobacco Ext 1.4 extensin gene upon mechanical constraint and localization of regulatory regions. *Plant Biology* **3**(1): 32-41.

Scheller HV, Ulvskov P. 2010. Hemicelluloses. *Annual Review of Plant Biology, Vol 61* **61**: 263-289.

Schols HA, Geraeds CCJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ. 1990. Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. *Carbohydrate Research* **206**(1): 105-115.

Schols HA, Bakx EJ, Schipper D, Voragen AGJ. 1995. A xylogalacturonan subunit present in the modified hairy regions of apple pectin. *Carbohydrate Research* **279**: 265-279.

Schols HA, Voragen AGJ, Visser J, Voragen AGJ. 1996. Complex Pectins: Structure elucidation using enzymes. *Progress in Biotechnology* Elsevier, Volume 14: 3-19.

Schultz CJ, Johnson KL, Currie G, Bacic A. 2000. The classical arabinogalactan protein gene family of arabidopsis. *Plant Cell* **12**(9): 1751-1767.

Schultz CJ, Rumsewicz MP, Johnson KL, Jones BJ, Gaspar YM, Bacic A. 2002. Using genomic resources to guide research directions. The arabinogalactan protein gene family as a test case. *Plant Physiology* **129**(4): 1448-1463.

Seifert GJ, Roberts K. 2007. The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58**: 137-161.

Sheng J, D'Ovidio R, Mehdy MC. 1991. Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. *Plant Journal* **1**(3): 345-354.

Showalter AM. 1993. Structure and function of plant cell wall proteins. *Plant Cell* **5**(1): 9-23.

Showalter AM, Keppler B, Lichtenberg J, Gu D, Welch LR. 2010. A Bioinformatics Approach to the Identification, Classification, and Analysis of Hydroxyproline-Rich Glycoproteins. *Plant Physiology* **153**(2): 485-513.

Sitrit Y, Hadfield KA, Bennett AB, Bradford KJ, Downie AB. 1999. Expression of a polygalacturonase associated with tomato seed germination. *Plant Physiology* **121**(2): 419-428.

Sobry S, Havelange A, Van Cutsem P. 2005. Immunocytochemistry of pectins in shoot apical meristems: consequences for intercellular adhesion. *Protoplasma* **225**(1-2): 15-22.

Somerville C. 2006. Cellulose synthesis in higher plants. *Annual Review of Cell and Developmental Biology*, **22**: 53-78.

Sørensen SO, Pauly M, Bush M, Skjot M, McCann MC, Borkhardt B, Ulvskov P. 2000. Pectin engineering: Modification of potato pectin by in vivo expression of an endo-1,4-beta-D-galactanase. *Proceedings of the National Academy of Sciences of the United States of America* **97**(13): 7639-7644.

Sterling JD, Atmodjo MA, Inwood SE, Kolli VSK, Quigley HF, Hahn MG, Mohnen D. 2006. Functional identification of an Arabidopsis pectin biosynthetic homogalacturonan galacturonosyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **103**(13): 5236-5241.

Sugiyama J, Persson J, Chanzy H. 1991. Combined infrared and electron diffraction study of the polymorphism of native celluloses. *Macromolecules* **24**(9): 2461-2466.

Supapvanich S, Tucker GA. 2013. Cell Wall Hydrolysis in Netted Melon Fruit (Cucumis melo var. reticulatus L. Naud) during Storage. *Chiang Mai Journal of Science* **40**(3): 447-458.

Tan L, Qiu F, Lamport DTA, Kieliszewski MJ. 2004. Structure of a hydroxyproline (Hyp)-arabinogalactan polysaccharide from repetitive Ala-Hyp expressed in transgenic Nicotiana tabacum. *Journal of Biological Chemistry* **279**(13): 13156-13165.

Tan L, Eberhard S, Pattathil S, Warder C, Glushka J, Yuan CH, Hao ZY, Zhu X, Avci U, Miller JS, Baldwin D, Pham C, Orlando R, Darvill A, Hahn MG, Kieliszewski MJ, Mohnen D. 2013. An Arabidopsis Cell Wall Proteoglycan Consists of Pectin and Arabinoxylan Covalently Linked to an Arabinogalactan Protein. *Plant Cell* **25**(1): 270-287.

Taniguchi Y, Ono A, Sawatani M, Nanba M, Kohno K, Usui H, Kurimoto M, Matuhasi T. 1995. Cry-j-i, a major allergen of japanese cedar pollen, has pectate lyase enzyme-activity. *Allergy* **50**(1): 90-93.

Thibault JF, Renard CMGC, Axelos MAV, Roger P, Crépeau M-J. 1993. Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research* **238**(0): 271-286.

Verhertbruggen Y, Marcus SE, Haeger A, Ordaz-Ortiz JJ, Knox JP. 2009a. An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydrate Research* **344**(14): 1858-1862.

Verhertbruggen Y, Marcus SE, Haeger A, Verhoef R, Schols HA, McCleary BV, McKee L, Gilbert HJ, Knox JP. 2009b. Developmental complexity of arabinan polysaccharides and their processing in plant cell walls. *Plant Journal* **59**(3): 413-425.

Vincken JP, York WS, Beldman G, Voragen AGJ. 1997. Two general branching patterns of xyloglucan, XXXG and XXGG. *Plant Physiology* **114**(1): 9-13.

Vincken JP, Schols HA, Oomen RJ, McCann MC, Ulvskov P, Voragen AG, Visser RG. 2003. If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiology* **132**(4): 1781-1789.

Voragen AGJ, Pilnik W, Thibault JF, Axelos MAV, Renard CMGC. 1995. Food Polysaccharides and their Applications Marcel Dekker, New York: 287-339.

Voragen AGJ, Coenen G-J, Verhoef RP, Schols HA. 2009. Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry* **20**(2): 263-275.

Vriesmann LC, Petkowicz CLO. 2013. Highly acetylated pectin from cacao pod husks (Theobroma cacao L.) forms gel. *Food Hydrocolloids* **33**(1): 58-65.

Wang H, Guo Y, Lv F, Zhu H, Wu S, Jiang Y, Li F, Zhou B, Guo W, Zhang T. 2010. The essential role of GhPEL gene, encoding a pectate lyase, in cell wall loosening by depolymerization of the de-esterified pectin during fiber elongation in cotton. *Plant Molecular Biology* **72**(4-5): 397-406.

Willats WGT, Marcus SE, Knox JP. 1998. Generation of a monoclonal antibody specific to (1-->5)-α-L-arabinan. *Carbohydrate Research* **308**(1-2): 149-152.

Willats WGT, Gilmartin PM, Mikkelsen JD, Knox JP. 1999a. Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a naive phage display library. *Plant Journal* **18**(1): 57-65.

Willats WGT, Steele-King CG, Marcus SE, Knox JP. 1999b. Side chains of pectic polysaccharides are regulated in relation to cell proliferation and cell differentiation. *Plant Journal* **20**(6): 619-628.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001a. Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology* **47**(1): 9-27.

Willats, W.G., Orfila, C., Limberg, G., Buchholt, H.C., van Alebeek, G.J., Voragen, A.G., Marcus, S.E., Christensen, T.M., Mikkelsen, J.D., Murray, B.S., Knox, J.P., 2001b. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *Journal of Biological Chemistry* **276**(22), 19404-19413.

Willats WGT, McCartney L, Steele-King C, Marcus S, Mort A, Huisman M, Alebeek G-J, Schols H, Voragen AJ, Goff A, Bonnin E, Thibault J-F, Knox JP. 2004. A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta* **218**(4): 673-681.

Willats WGT, Knox JP, Mikkelsen JD. 2006. Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology* **17**(3): 97-104.

Wolf S, Mouille G, Pelloux J. 2009. Homogalacturonan Methyl-Esterification and Plant Development. *Molecular Plant* **2**(5): 851-860.

Wong D. 2008. Enzymatic deconstruction of backbone structures of the ramified regions in pectins. *Protein Journal* **27**(1): 30-42.

Wu H, de Graaf B, Mariani C, Cheung AY. 2001. Hydroxyproline-rich glycoproteins in plant reproductive tissues: structure, functions and regulation. *Cellular and Molecular Life Sciences* **58**(10): 1418-1429.

Wyatt RE, Nagao RT, Key JL. 1992. Patterns of soybean proline-rich protein gene expression. *Plant Cell* **4**(1): 99-110.

Yapo BM, Lerouge P, Thibault J-Fo, Ralet M-C. 2007. Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydrate Polymers* **69**(3): 426-435.

Yapo BM. 2011. Pectic substances: From simple pectic polysaccharides to complex pectins- A new hypothetical model. *Carbohydrate Polymers* **86**(2): 373-385.

York WS, Darvill AG, Albersheim P. 1984. Inhibition of 2,4-dichlorophenoxyacetic acid-stimulated elongation of pea stem segments by a xyloglucan oligosaccharide. *Plant Physiology* **75**(2): 295-297.

York WS, Kolli VSK, Orlando R, Albersheim P, Darvill AG. 1996. The structures of arabinoxyloglucans produced by solanaceous plants. *Carbohydrate Research* **285**: 99-128.

Zaidel DNA, Meyer AS. 2012. Biocatalytic cross-linking of pectic polysaccharides for designed food functionality: Structures, mechanisms, and reactions. *Biocatalysis and Agricultural Biotechnology* **1**(3): 207-219.

Zandleven J, Beldman G, Bosveld M, Schols HA, Voragen AGJ. 2006. Enzymatic degradation studies of xylogalacturonans from apple and potato, using xylogalacturonan hydrolase. *Carbohydrate Polymers* **65**(4): 495-503.

Zykwinska A, Ralet MC, Garnier CD, Thibault JF. 2005. Evidence for in vitro binding of pectin side chains to cellulose. *Plant Physiology* **139**(1): 397-407.

Zykwinska A, Rondeau-Mouro C, Garnier C, Thibault JF, Ralet MC. 2006. Alkaline extractability of pectic arabinan and galactan and their mobility in sugar beet and potato cell walls. *Carbohydrate Polymers* **65**(4): 510-520.

Zykwinska A, Thibault JF, Ralet MC. 2007. Organization of pectic arabinan and galactan side chains in association with cellulose microfibrils in primary cell walls and related models envisaged. *Journal Experimental of Botany* **58**(7): 1795-1802.

CHAPTER II

Wine RGII sub-domains recovery and fine structure variability

II.1 Introduction

Pectin is a major constituent of primary cell walls of gymnosperm and dicotyledon (O'Neill *et al.*, 2004). It is a multi-component polymer consisting of rhamnogalacturonan I (RGI), homogalacturonan (HG) and HG analogues; these sub-structures are covalently linked to each other (Ishii and Matsunaga, 2001; Ridley *et al.*, 2001; Willats *et al.*, 2001; Mohnen, 2008). The backbone structure of RGI is a repeating dimer of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]. This heteropolymer is substituted by arabinan, galactan and arabinogalactan side chains in variable amounts (Willats *et al.*, 2001). HG is a linear polymer of (1→4) linked α-D-Gal*p*A residues, which are usually methyl-esterified at *O*-6 and sometimes acetyl-esterified at *O*-2 and/or *O*-3 (Mohnen, 2008). HG analogues contain a (1→4) linked α-D-Gal*p*A backbone substituted by sugar monomers, sugar dimers or complex side chains. In xylogalacturonan and apiogalacturonan, the Gal*p*A units are branched at *O*-3 and *O*-2 by monomers or dimers of xylose and apiose residues, respectively (Caffall and Mohnen, 2009).

Rhamnogalacturonan II (RGII) contains five different side chains, defined as A-E, which decorate a short Gal*p*A backbone. This very complex molecule is composed of more than 12 different sugars and 20 different linkages (O'Neill *et al.*, 2004) constituting a mega-oligosaccharide of 5-10 kDa (Whitcombe *et al.*, 1995; Vidal *et al.*, 2000; Strasser and Amadò, 2001). RGII represents 1-5% of dicotyledon primary cell walls (Darvill *et al.*, 1978; O'Neill *et al.*, 1990; Matoh *et al.*, 1996). RGII was first characterised in sycamore by Darvill and co-workers in 1978. The extraction method involved treatment with endo-polygalacturonase in order to release RGII from HG (Ishii and Matsunaga, 2001). Fractionation of the hydrolysate by size-exclusion chromatography (SEC) combined with anion-exchange chromatography (AEC) allowed RGII purification. Similar methods using various pectolytic enzymes and chromatographic methods have been used for RGII recovery from rice (Thomas *et al.*, 1989), bamboo (Kaneko *et al.*, 1997), tobacco BY-2 cells (Kobayashi *et al.*, 1997), *Cryptomeria japonica* (Edashige and Ishii, 1998), ginseng (Shin *et al.*, 1998), *Chenopodium album* L. (Fleischer *et al.*, 1999), pumpkin (Ishii *et al.*, 2001), red beet (Strasser and Amadò, 2001), *Arabidopsis thaliana* (Glushka *et al.*, 2003), lycophytes (Matsunaga *et al.*, 2004), pteridophytes (Matsunaga *et al.*, 2004) and bryophytes (Matsunaga *et al.*, 2004). In wine, enzymatic degradation occurs during fermentation and the remaining non-degraded polysaccharides are mannoproteins from yeasts, arabinogalactan proteins (AGP), RGI and RGII, the latter representing up to 20% of the recovered wine polysaccharides (Doco and Brillouet, 1993; Pellerin *et al.*, 1996; Vidal *et al.*, 1999; Vidal *et al.*, 2003).

RGII structure has been determined using different approaches. This complex molecule encompasses specific sugar species, aceric acid (Ace*f*A), methyl fucose (*O*-Me-Fuc*p*), methyl xylose (*O*-Me-Xyl*p*), apiose (Api*f*), 3-deoxy-D-*manno*-2-octulosonic acid (Dha), 3-deoxy-D-*lyxo*-2-heptulosonic acid (Kdo), which can be detected and quantified (York *et al.*, 1985; Kobayashi *et al.*, 1996; Edashige and Ishii, 1998; Stevenson *et al.*, 1998; Doco *et al.*, 2001; Glushka *et al.*, 2003; Yapo *et al.*, 2007). Unusual linkages include 3-linked rhamnosyl, 3,4-linked fucosyl, 2-linked glucuronosyl residues and the fully substituted rhamnose are RGII-specific and can be detected by glycosyl linkage analysis (Darvill *et al.*, 1978; Thomas *et al.*, 1989; Kobayashi *et al.*, 1996; Pellerin *et al.*, 1996; Edashige and Ishii, 1998; Shimokawa *et al.*, 1999; Strasser and Amadò, 2001; Glushka *et al.*, 2003). RGII glycosyl sequence was mostly determined after chemical fragmentation of the molecule. Lithium dissolved in ethylamine

(Stevenson *et al.*, 1988; Edashige and Ishii, 1998), formolysis (Melton *et al.*, 1986) and Smith degradation (Puvanesarajah *et al.*, 1991; Glushka *et al.*, 2003) are different ways that have been used to recover RGII-derived oligosaccharides. However, partial degradation by mild acid hydrolysis – usually with trifluoroacetic acid (TFA) – has been the most widely used method to isolate the RGII components of plant cell walls (Spellman *et al.*, 1983; Stevenson *et al.*, 1988; Thomas *et al.*, 1989; Whitcombe *et al.*, 1995; Shin *et al.*, 1998; Matsunaga *et al.*, 2004; Reuhs *et al.*, 2004; Séveno *et al.*, 2009; Voxeur *et al.*, 2011; Pabst *et al.*, 2013). The recovered oligosaccharides have then been characterised by sugar and linkage analysis, or have been directly studied by mass spectrometry. Such oligosaccharides were also analysed by NMR to provide information about sugar sequence, anomeric configuration and the location of non-sugar substituents (Melton *et al.*, 1986; Puvanesarajah *et al.*, 1991; Whitcombe *et al.*, 1995; Glushka *et al.*, 2003; Pabst *et al.*, 2013). The proposed distribution of side chains, based on NMR measurements combined with molecular modelling, played a major role in the description of the RGII three-dimensional structure (Vidal *et al.*, 2000; Pérez *et al.*, 2003; Rodríguez-Carvajal *et al.*, 2003).

Around 95% of RGII molecules from normal plants are dimeric (O'Neill *et al.*, 2001), which was shown to involve in boron integration (O'Neill *et al.*, 1996; Fleischer *et al.*, 1999), sequestering about 80 % of the cellular boron in plants (Matoh *et al.*, 1996). Boron was shown to have a key role in plant growth, and RGII structure was shown to be essential for its internalization in the cell wall (O'Neill *et al.*, 2001). Boron deficiency leads to larger cells with swollen walls, and the RGII-dimer-boron complex has therefore been suspected to control cell wall properties (Matoh *et al.*, 2000).

Interestingly, the structurally highly complex RGII is highly conserved over plant evolution (Matsunaga *et al.*, 2004). However, characterisation of oligosaccharides generated by RGII partial hydrolysis highlighted several potential structural alterations (Matsunaga *et al.*, 2004; Pabst *et al.*, 2013). Differentiating intrinsic structural variations from structural variations due to the acid hydrolysis conditions used remains challenging. Herein we report on a range of sequential mild acid hydrolyses and their impact on wine RGII side chains, backbone recovery and fine structure. The potential influence of the observed structural variability *in muro* will be discussed.

II.2 Material and methods

II.2.a RGII extraction

Total colloids were recovered from 21 L of red wine (Merlot) as described by Vidal *et al.* (2003). The total colloids solution was mixed with one volume of strong cation-exchange resin (IR-120, Amberlite) and left overnight at room temperature. The unbound fraction was mixed with 1/2 volume of strong cation-exchange gel (Sepharose SP Fast Flow, GE Healthcare) and left 4 h at room temperature. The unbound fraction was finally mixed with one volume of strong anion-exchange resin (IRA-958, Amberlite) and left overnight at room temperature. The unbound fraction, corresponding to non-pigmented total wine polysaccharides, was dialyzed against distilled water and freeze-dried. Total wine polysaccharides (7.1 g) were solubilised in 25 mM sodium acetate buffer pH 4.5 (320 mL) and loaded at 90 mL/h in four batches on a DEAE-Sepharose Fast Flow column (5 x 15 cm) equilibrated with the same buffer. An unbound fraction was recovered and bound polysaccharides were eluted by steps of NaCl (50 mM and 250 mM in the starting buffer). The fraction eluted with 250 mM NaCl, corresponding to raw RGII, was dialyzed and freeze-dried. Raw RGII (3 g) was solubilised in 50 mM sodium acetate buffer pH 4.5 (60 mL) and loaded in twelve batches at 90 mL/h on a Sephacryl-S400 HR column (2.6 x 100 cm). Appropriate fractions were pooled, dialyzed and freeze-dried to yield 1.24 g of purified RGII.

II.2.b Sequential mild acid hydrolysis and anion-exchange chromatography

RGII (200 mg) was dissolved in 20 mL water and treated with 0.1 M TFA for 16 h at 40°C. TFA was removed by in vacuum rotatory evaporation at 40°C to dryness. The hydrolysate was solubilised in water and further evaporated to dryness. This last step was repeated three times. The hydrolysate was finally solubilised in water and pH was adjusted to 4.5 with a few drops of 100 mM NaOH. AEC was performed at room temperature on a DEAE-Sepharose Fast Flow column (1.6 x 13.5 cm) equilibrated with degased 25 mM sodium acetate buffer pH 4.5 at a flow rate of 90 mL/h. The hydrolysate (20 mL) was loaded onto the column and the gel was washed with 50 mL of 25 mM sodium acetate buffer pH 4.5. The bound material

was eluted with a linear NaCl gradient (0 - 100 mM NaCl in 25 mM sodium acetate buffer, 300 mL). Sodium acetate buffer containing 500 mM NaCl (50 mL) was then applied. Fractions (3.5 mL) were collected and analysed for their content of GalA and neutral sugars by colorimetry (Thibault, 1979; Tollier and Robin, 1979). Appropriate fractions were combined in pools and concentrated by vacuum rotary evaporation at 40 °C.

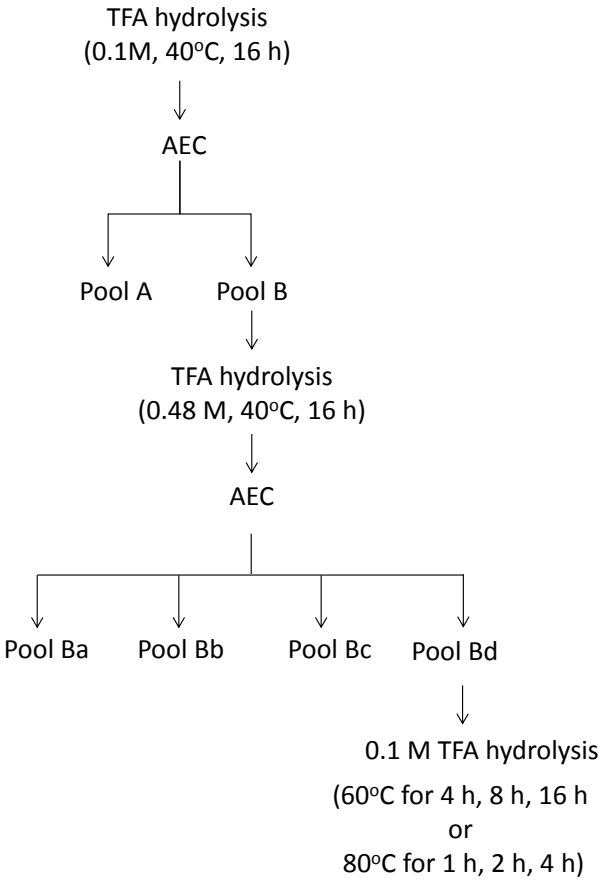


Figure II.1: RGII sequential extraction scheme

The pool eluted during the washing step (Pool A) was desalted using a column (1.6 x 100 cm) of Sephadex G-10 run in deionised water at 1 mL/min. The pool eluted with 500 mM NaCl (pool B) was dialysed, freeze-dried, and further hydrolyzed with 0.48 M TFA for 16 h at 40°C. TFA was removed and pH was adjusted as described above. After fractionation by AEC, pools eluted during the washing step or by the linear NaCl gradient (Pools Ba, Bb and Bc) were desalted using a column of Sephadex G-10 as described above. The pool eluted at 500 mM NaCl (pool Bd) was dialysed and freeze-dried. Pool Bd aliquots (0.25 mg) were further

hydrolyzed with 1 mL 0.1 M TFA for 4h, 8h or 16h at 60°C or for 1h, 2h or 4h at 80°C (Figure.I.1). TFA was removed as described above and hydrolysates were solubilised in 1 mL water.

II.2.c Alkaline hydrolysis

Samples (100 µg in 400 µL water) were incubated with 25 mM heptylamine (HA, Sigma-Aldrich) in a 1:1 ratio (v/v) for 3 days. A tri-methyl-esterified tri-Gal*p*A standard was incubated under the same conditions as a positive control.

II.2.d Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

MALDI-TOF-MS spectra were acquired on an Autoflex III TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a Smartbeam Laser (355 nm, 200 Hz) and a reflector detection. Freeze-dried samples were solubilised in water (1 mg/mL). Sample solution (1 mg/mL water or HA hydrolysates) and matrix, prepared as previously described (Ropartz *et al.*, 2011), were mixed (1/1, v/v) and applied on a polished steel MALDI target plate. Spectra were recorded using FlexControl and processed using FlexAnalysis (Bruker Daltonics). Mass spectra were acquired in positive and negative ionisation mode.

II.3 Results

II.3.a Side chain debranching

RGII is a substituted α-D-Gal*p*A backbone carrying five structurally distinct side chains: (i) the octameric side chain A encompassing three acidic sugars, (ii) the hexameric to nonameric side chain B encompassing one acidic sugar, (iii) the dimeric side chains C and D encompassing one acidic sugar, and (iv) the monomeric neutral side chain E. The monomeric side chain E (α-L-Ara*f*), when present, is linked *O*-3 to the Gal*p*A backbone (Melton *et al.*,

1986; Pérez *et al.*, 2003). The two dimeric side chains C and D are linked to the backbone at *O*-3 *via* their ulosonic acid residue (Stevenson *et al.*, 1988). The complex side chains A and B are both attached at *O*-2 of the backbone *via* an apiosyl residue (Stevenson *et al.*, 1988). The relative position of the side chains on the Gal*p*A backbone is not fully understood (Yapo, 2011). The three side chains C, B and D are located, in this order, to the first five Gal*p*A residues from the reducing end of the molecule (Vidal *et al.*, 2000). Side chain A is expected to be the last side chain present from the reducing end (Vidal *et al.*, 1999). Since, glycosyl linkage analyses detected the presence of (2,3,4) linked Gal*p*A residues in different species, two side chains are likely to be linked onto the same Gal*p*A unit (Doco and Brillouet, 1993; Pellerin *et al.*, 1996; Doco *et al.*, 1997; Strasser and Amadò, 2001). Side chain E has not been precisely located to date but could be linked to the third Gal*p*A residue from the non-reducing end together with side chain A (Yapo, 2011).

Table II.1: Hydrolysis conditions used for RGII side chain release; 0.1 M TFA was used in all studies

Fragment released	Incubation time (h)	Temperature (°C)	Degraded products	References
C and D	0.5	60	-	Thomas *et al.*, 1989
B	16	40	+/-	Whitcombe *et al.* 1995
B	16	40	+/-	Matsunaga *et al.* 2004
B	16	40	+/-	Séveno *et al.* 2009
B	16	40	+	Voxeur *et al.* 2011
B	24	40	+/-	Shin *et al.* 1998
B	24	40	+/-	Reuhs *et al.* 2004
B	24	50	+	Thomas *et al.* 1989
B	1	80	++	Pabst *et al.* 2013
B	1	80	+++	Séveno *et al.* 2009
B	1	100	++++	Séveno *et al.* 2009
A	16	40	-	Séveno *et al.* 2009
A	16	40	+/-	Voxeur *et al.* 2011
A	24	50	+	Thomas *et al.* 1989
A	1	80	+/-	Pabst *et al.* 2013
A	1	80	++	Séveno *et al.* 2009
A	1	100	+++	Séveno *et al.* 2009

Refers to the relative amounts of degradation products observed (from -, very low amounts to ++++, very high amounts).

Debranching the various side chains from the Gal*p*A backbone required different hydrolysis conditions. Their ability to be detached is likely ranking C-D>B>A (Table.II.1). The acid-lability of side chain E is presently unknown. Hydrolysis conditions have to be carefully chosen to avoid side chain degradation. Dimeric side chains C and D can both be detached with 0.1 M TFA for 6 h at 40°C (York *et al.*, 1985). When the same hydrolysis conditions (0.1 M TFA at 40°C) were applied for a longer time (16 or 24 h), side chain B was detached from the Gal*p*A backbone and only trace amounts of degradation products were observed (Table.II.1). When more drastic conditions were used, extensive hydrolysis of chain B was reported (Séveno *et al.*, 2009). In contrast, Pabst *et al.* (2013) found no significant difference in the data whether the hydrolysis was performed for 16 h at 40°C or for 1 h at 80°C. Side chain A was efficiently released from the RGII backbone by increasing the temperature of hydrolysis to 80°C for 1 h but some degradation products were observed (Table.II.1). Acid-lability is therefore specific for each branching residue, and sequential hydrolysis is highly recommended for the recovery of RGII side chains (Séveno *et al.*, 2009).

To separate hydrolysis products, various chromatographic methods have been used. SEC has been widely used for side chain B purification, the detached side chain B (DP 6-9) being well separated from detached dimeric side chains C and D and from RGII remnants consisting of side chain A attached to the Gal*p*A backbone (Spellman *et al.*, 1983; Whitcombe *et al.*, 1995; Shin *et al.*, 1998; Matsunaga *et al.*, 2004; Reuhs *et al.*, 2004). AEC has also been used to separate hydrolysis products based on their charges that are not equally distributed over the entire molecule; the side chains and backbone (Thomas *et al.*, 1989). Separation on porous graphitized carbon liquid chromatography was recently been performed at analytical scale (Pabst *et al.*, 2013).

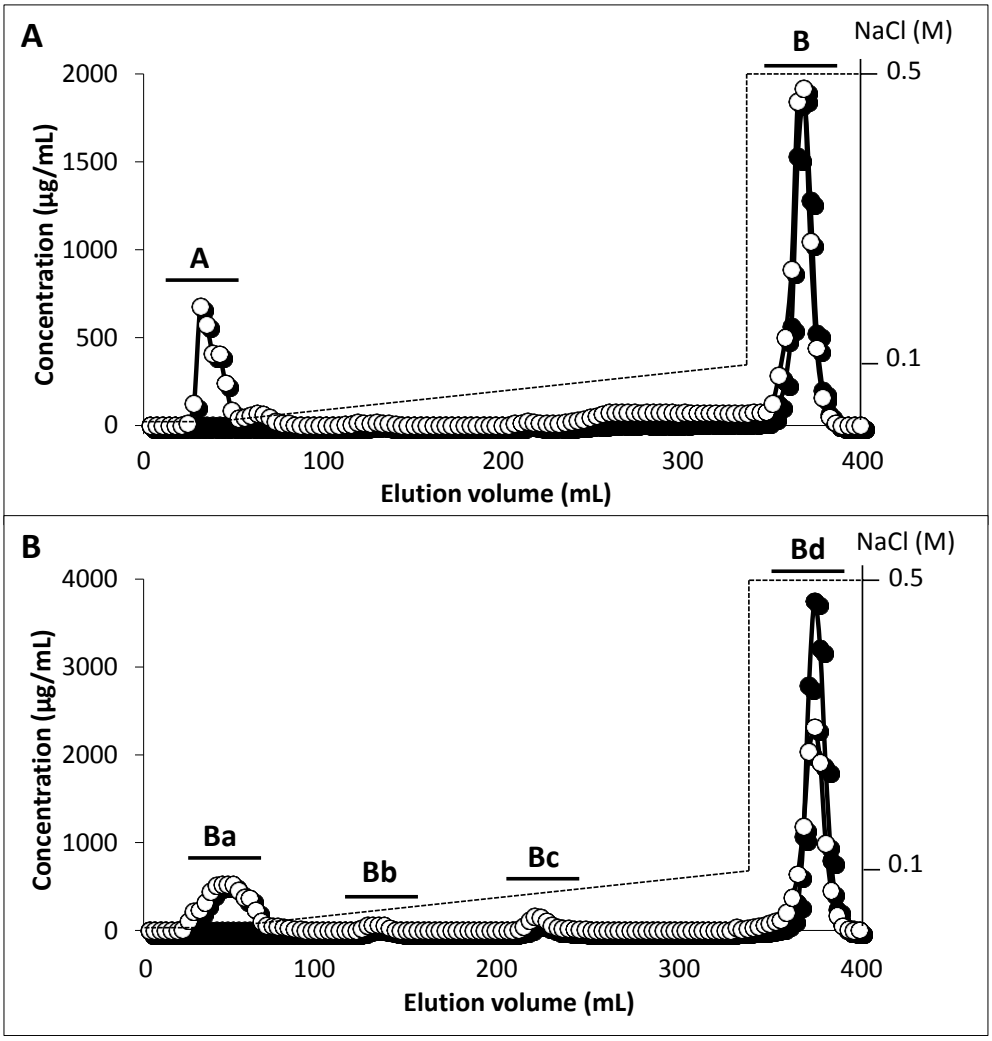


Figure II.2: Anion-exchange chromatography elution patterns of RGII hydrolysates. (A) Wine RGII hydrolyzed by 0.1 M TFA for 16 h at 40°C. (B) Pool Bd further hydrolyzed with 0.48 M TFA for 16 h at 40°C. ○ neutral sugar; ● uronic acid.

In the present study, wine RGII was submitted to sequential acid hydrolysis and hydrolysis products were separated by AEC. After a first hydrolysis step (0.1 M TFA, 16 h, 40°C), two populations were separated (Figure.II.2A). Sugar analysis revealed that Pool A did not contain Gal*p*A. Side chains B, C and D lack uronic acids, and were likely to be released under the acid conditions used (Table.II.1), were probably present in this pool. Pool B, which contained both neutral sugars and Gal*p*A, was eluted with an ionic strength compatible with the presence of more than seven charges, and was expected to contain intact and/or partially hydrolyzed RGII. Pool B underwent a second hydrolysis step under slightly harsher conditions (0.48 M TFA, 16 h, 40°C), and AEC of the hydrolysate generated four populations (Figure.II.2B). Pool Ba was similar to Pool A with respect to elution volume and global sugar

analysis and probably contains side chains B, C and D that were not released during the first acidic treatment (Spellman *et al.*, 1983; Whitcombe *et al.*, 1995; Matsunaga *et al.*, 2004). Pool Bb and Pool Bc both contained GalA and neutral sugars and were eluted with ionic strengths compatible with the presence of two and three charges, respectively. Pool Bd was expected, as the previously recovered Pool B, to contain RGII remnants that were partially hydrolyzed. Detection of neutral sugars in this pool provides evidence that side chains are not totally released after the second hydrolysis step.

II.3.b Side chain B

Side chain B was first characterised as a heptasaccharide in RGII from sycamore suspension cultured cells (Spellman *et al.*, 1983). However, some variability with respect to side chain B degree of polymerization (DP) was observed, and side chains B of DP 6 to DP 9 have been purified from various plant sources (Table.II.2; Figure.II.3). The length of side chain B depends on variations in glycosyl substitution on the α-L-Ara*p* residue (Figure.II.3). The heptasaccharide described by Spellman *et al.* (1983) is rhamnosylated at *O*-2 of the α-L-Ara*p* residue (Figure.II.3). This structure has been detected in most plants (Table.II.2). Two different forms of octasaccharides were also found. The first form consists of a disaccharide α-L-Rha*p*-(2→1)-β-L-Ara*f* substitution at *O*-2 of the α-L-Ara*p* residue. This type of octasaccharide was detected in red wine, bamboo shoot, suggi and also in sycamore in the latest studies (Stevenson *et al.*, 1988; Whitcombe *et al.*, 1995; Kaneko *et al.*, 1997; Edashige and Ishii, 1998; Glushka *et al.*, 2003; Pabst *et al.*, 2013). In the second octameric form, α-L-Ara*p* was di-substituted at *O*-2 and *O*-3 by α-L-Rha*p* residues. This structure has been detected in *Arabidopsis thaliana* and in some lycophytes and pteridophytes (Matsunaga *et al.*, 2004). A nonasaccharide containing both the disaccharide α-L-Rha*p*-(2→1)-β-L-Ara*f* substitution at *O*-2 of α-L-Ara*p* and monomeric α-L-Rha*p* at *O*-3 of the α-L-Ara*p* residue was identified in red wine and ginseng (Shin *et al.*, 1998; Glushka *et al.*, 2003; Pabst *et al.*, 2013). A hexasaccharide resulting from the absence of rhamnosylation of the α-L-Ara*p* residue was also detected in sugi, red beet and *Arabidopsis thaliana* (Edashige and Ishii, 1998; Strasser and Amadò, 2001; Pabst *et al.*, 2013). Different structures are supposed to co-exist in the

same plant and it was recently shown that their proportion varies during plant development and is organ-specific (Pabst *et al.*, 2013).

Table II.2: RGII side chain B structural variability with respect to plant sources

Plant source	α-L-Rha*p* linked at *O*-3 of α-L-Ara*p*	α-L-Rha*p* linked at *O*-2 of α-L-Ara*p*	β-L-Ara*f* linked at *O*-2 of α-L-Rha*p*	dp	References
Sycamore	-	+	+/-	7-8	Spellman *et al.,* 1983; Stevenson *et al.*, 1988; Whitcombe *et al.*, 1995
Red wine	+/-	+/-	+/-	6-9	Glushka *et al.*, 2003; Pabst *et al.*, 2013
Panax ginseng	+	+	+	9	Shin *et al.*, 1998
Pectinol AC	-	-	-	6	Stevenson *et al.*, 1988
Arabidopsis thaliana	+/-	+/-	-	6-8	Glushka *et al.*, 2003; Pabst *et al.*, 2013
Rice	-	+	-	7	Thomas *et al.*, 1989
Bamboo	-	+	+/-	7-8	Kaneko *et al.*, 1997
Red beet	-	+/-	-	6-7	Strasser and Amadò, 2001
Sugi	-	+/-	+/-	6-8	Edashige and Ishii, 1998
Lycophyte/ pteridophyte	+/-	+/-	-	7-8	Matsunaga *et al.*, 2004

- absent; +/- partly present ; + present

Variation in esterification and etherification status was also detected in side chain B. In some lycophytes and pteridophytes; terminal Rha*p* units linked *O*-2 and/or *O*-3 to the α-L-Ara*p* residue contained were shown to contain 3-*O*-methyl groups (Matsunaga *et al.*, 2004). Acetyl groups linked to α-L-Ace*f*A and/or 2-*O*-Me-α-D-Fuc*p* were also identified (Whitcombe *et al.*, 1995). Mono-, di- and no-acetylation generally co-exist in all species although mono-acetylation on the 2-*O*-Me-α-D-Fuc*p* residue appears to be the predominant form (Spellman *et al.*, 1983; Whitcombe *et al.*, 1995). In *Arabidopsis thaliana* RGII acetylation was reported not to be organ specific (Pabst *et al.*, 2013).

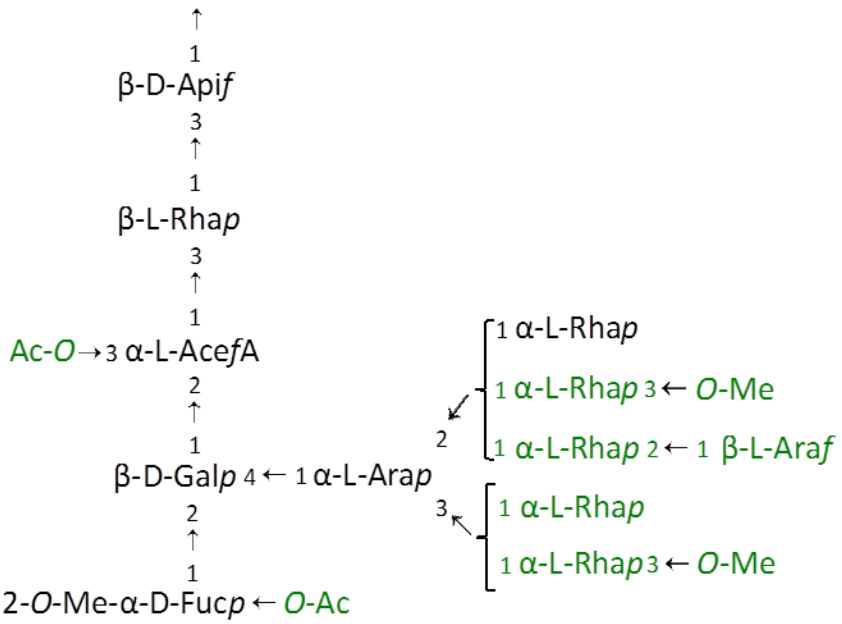


Figure II.3: Side chain B structure. Sugars in black were detected by Spellman *et al*. (1983). Acethyl groups can esterify the 2-*O*-α-D-Me-Fuc*p* and/or α-L-Ace*f*A residue. Methyl groups can esterify the α-L-Rha*p* residues at *O*-3.

Pool A and Ba recovered after AEC (Figure.II.2) were analysed by mass spectrometry (Figure.II.4). No signal was detected for *m/z* < 1000. Pool A arising from 0.1 M TFA, 40°C, 16 h-treated RGII exhibited one main signal at *m/z* 1375 corresponding to a mono-acetylated side chain B of DP 9 (Figure.II.4A). All other signals were very weak. Signals at *m/z* 1333 and 1417 were assigned to non-acetylated and di-acetylated side chain B of DP 9. Signals at *m/z* 1243 and 1229 were assigned to acetylated side chain B of DP 8 having lost a β-L-Ara*f* residue or α-L-Rha*p* unit, respectively. The presence of a signal at *m/z* 1097 indicates that acetylated side chain B of DP 7 had lost the terminal disaccharide α-L-Rha*p*-(2→1)-β-L-Ara*f* or both the terminal β-L-Ara*f* and α-L-Rha*p* units. Pool Ba arising from RGII treated with 0.48 M TFA at 40°C for 16 h exhibited a more complex spectrum (Figure.II.4B). The ion corresponding to the di-acetylated side chain B of DP 9 was not detected. The two main signals at *m/z* 1375 and 1333 were assigned to mono-acetylated and non-acetylated side chain B of DP 9, respectively. Signals at *m/z* 1243, 1229 and 1097 corresponded to acetylated side chain B lacking a terminal β-L-Ara*f* , terminal α-L-Rha*p* or the disaccharide (α-L-Rha*p*-(2→1)-β-L-Ara*f*), respectively. Similar degradation products arising from non-acetylated side chain B were observed at *m/z* 1201, 1187 and 1055. Both non-acetylated and mono-acetylated side chain B of DP 8 having lost one β-L-Ara*f* residue were also observed and gave

strong signals. Signals at *m/z* 1173 and 1041 were assigned to non-acetylated side chain B fragments having lost a 2-*O*-Me-α-L-Fuc*p* residue or both a 2-*O*-Me-α-L-Fuc*p* and a single β-L-Ara*f* unit, respectively. The acetylated form of these two fragments was not detected. It is therefore assumed that the acetyl group is preferably located on the 2-*O*-Me-α-L-Fuc*p* residue as previously reported (Whitcombe *et al.*, 1995). It is evident that the harsher hydrolysis conditions applied for side chain B generated new fragments indicating that the β-L-Ara*f*, 2-*O*-Me-α-L-Fuc*p* and acetyl linkages were particularly acid-labile. In contrast, the rhamnosylation status of side chain B was not greatly affected by hydrolysis conditions; the signal at *m/z* 1229, corresponding to side chain B lacking one Rha*p* residue, was weak irrespective of the extraction condition (Figure.II.4).

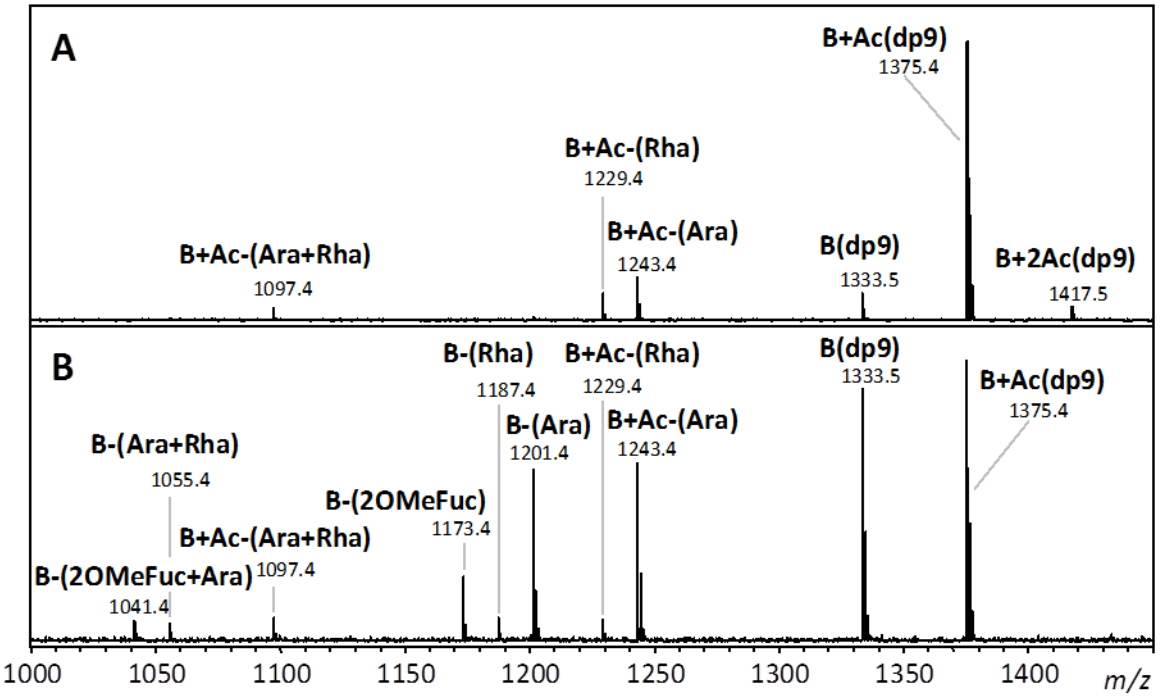


Figure II.4: MALDI-TOF mass spectra (negative mode) of the RGII hydrolysates. (A) Pool A recovery after AEC of 0.1 M TFA for 16 h at 40°C. (B) Pool Bd recovered after AEC of 0.48 M TFA for 16 h at 40°C.

II.3.c Side chain A

Side chain A was first characterised as an octasaccharide (Figure.II.5) in sycamore and rice (Stevenson *et al.*, 1988; Thomas *et al.*, 1989). Side chain A is particularly important since it is involved in RGII dimerization *via* a boron di-ester bond (Shimokawa *et al.*, 1999). Boron is covalently linked to β-D-Api*f* units at *O*-2 and *O*-3 (Ishii and Ono, 1999; O'Neill *et al.*, 1996).

This occurs only with Api*f* residues from side chain A that has less conformational flexibility than side chain B (Glushka *et al.*, 2003; Rodríguez-Carvajal *et al.*, 2003; O'Neill *et al.*, 2004). Side chain A also contains three uronic acids that constitute a suitable site for chelating cations (Pérez *et al.*, 2003). Two calcium ions, which do not interact with boron, are indeed required to stabilise the RGII complex (O'Neill *et al.*, 1996; Matoh and Kobayashi, 1998; Kobayashi *et al.*, 1999). Possible methylation of side chain A was first hypothesized by Séveno *et al*. (2009). This was recently confirmed by Pabst *et al.* (2013) who showed that both methyl-esterification at *O*-6 of the β-D-Glc*p*A residue and methylation at *O*-3 and/or *O*-4 of the β-D-Gal*p*A residue could occur. Methyl-esterification is likely to affect calcium-mediated RGII complex stabilisation.

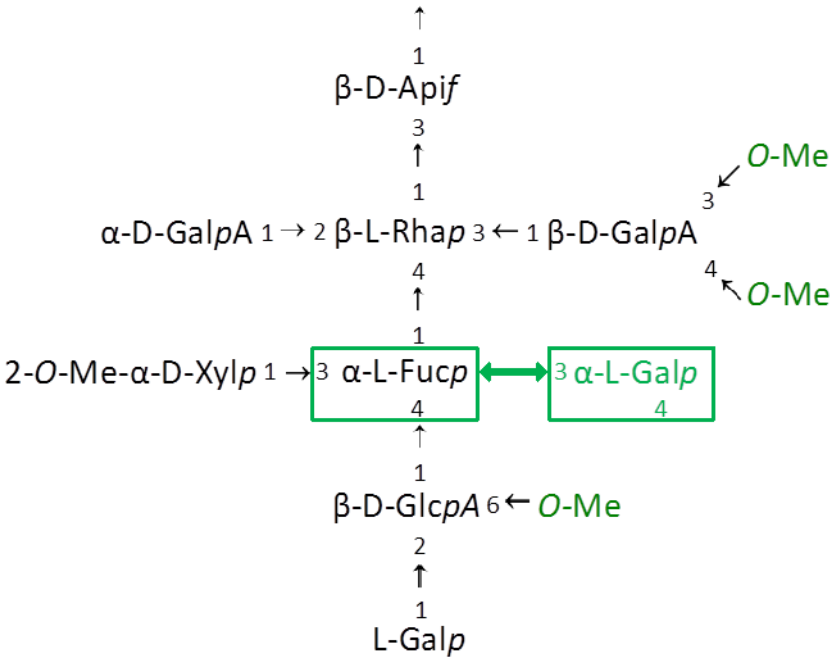


Figure II.5. Side chain A structure. The β-D-Glc*p*A residue can be methyl-esterified (in green). The β-D-Gal*p*A residue can be singly or doubly methyl-etherified (in green). The α–L-Fuc*p* residue can be oxidised in α–L-Gal*p* (green box).

Pool Bb and Bc from AEC (Figure.II.2B) were analysed by mass spectrometry (Figures.II.6A and .II.6C). No signal was detected at *m/z* < 1300. For Pool Bb, a major $(M+Na)^+$ ion corresponding to a mono-methylated intact chain A was detected at *m/z* 1315 (Figure.II.6A). No signal at *m/z* 1301, corresponding to a non-methylated full chain A was detected whereas ions at *m/z* 1329 and 1343, corresponding to di- and tri-methylated full chain A, respectively, were observed. For Pool Bc, a major $(M+Na)^+$ ion corresponding to a

non-methylated full chain A was detected at *m/z* 1301 (Figure.II.6C). Weak signals at *m/z* 1315 and 1329, corresponding to mono- and di-methylated chain A, respectively, were also detected. Pools Bb and Bc were incubated with heptylamine (HA), an alkaline component that can hydrolyse methyl-ester groups. A tri-methyl-esterified tri-Gal*p*A standard was incubated in the same conditions. Only non-methyl-esterified tri-Gal*p*A was detected by mass spectrometry after a 3 day-incubation. After HA treatment of Pool Bd, a shift from the mono-methylated (*m/z* 1315) to the non-methylated (*m/z* 1301) form of side chain A was observed (Figure.II.6B). This, together with the fact that Pool Bd was eluted by AEC for an ionic strength compatible with the presence of two charges, strongly supports the hypothesis that side chain A is mainly singly methyl-esterified in this pool. The fact that ions corresponding to mono- and di-methylated side chain A (*m/z* 1315 and 1329, respectively) can still be detected after HA treatment suggests that one or two methyl-etherification can exist in addition to methyl-esterification, as recently reported by Pabst *et al.* (2013). This hypothesis is supported by the disappearance of the signal assigned to tri-methylated side chain A (*m/z* 1343) (one methyl-ester and two methyl-ether groups) after HA treatment. In contrast, the Pool Bc spectrum remained similar after HA treatment, only lower intensities were observed due to the presence of HA (Figure.II.6D). Non-methylated, mono-methylated and di-methylated forms of side chain A were present in similar proportions before and after treatment. In this pool, methyl-etherification only is likely to be present, in agreement with the fact that Pool Bc was eluted by AEC for an ionic strength compatible with the presence of three charges.

Oxidation of the α-L-Fuc*p* or α-L-Gal*p*, adding 16 Da to side chain A, was recently observed by Pabst *et al.* (2013). Signals 16 Da greater than RGII-derived oligosaccharides were observed in Pool Bb and Bc (Figures.II.6A and .II.6C). Methyl-esterified and/or methyl-etherified forms of the oxidized structure were also evident. In Pool Bb the oxidized structure was only mono- (*m/z* 1331) and di-methylated (*m/z* 1345) (Figure.II.6A). After incubation with HA, ions at *m/z* 1317 and *m/z* 1331 assigned to non-methylated and mono-methylated oxidized side chain A, respectively, were observed (Figure.II.6B). In this pool, oxidized side chain A was therefore mono-methyl-esterified and non- or mono-methyl-etherified. Pool Bc, signals at *m/z* 1317 and 1331 were assigned to non-, and mono-methylated oxidized side chain A, respectively (Figure.II.6C). After HA treatment, no significant changes were observed in the

MS spectrum (Figure.II.6D). In this pool, oxidized side chain A species are either mono-methyl-etherified or non-methylated.

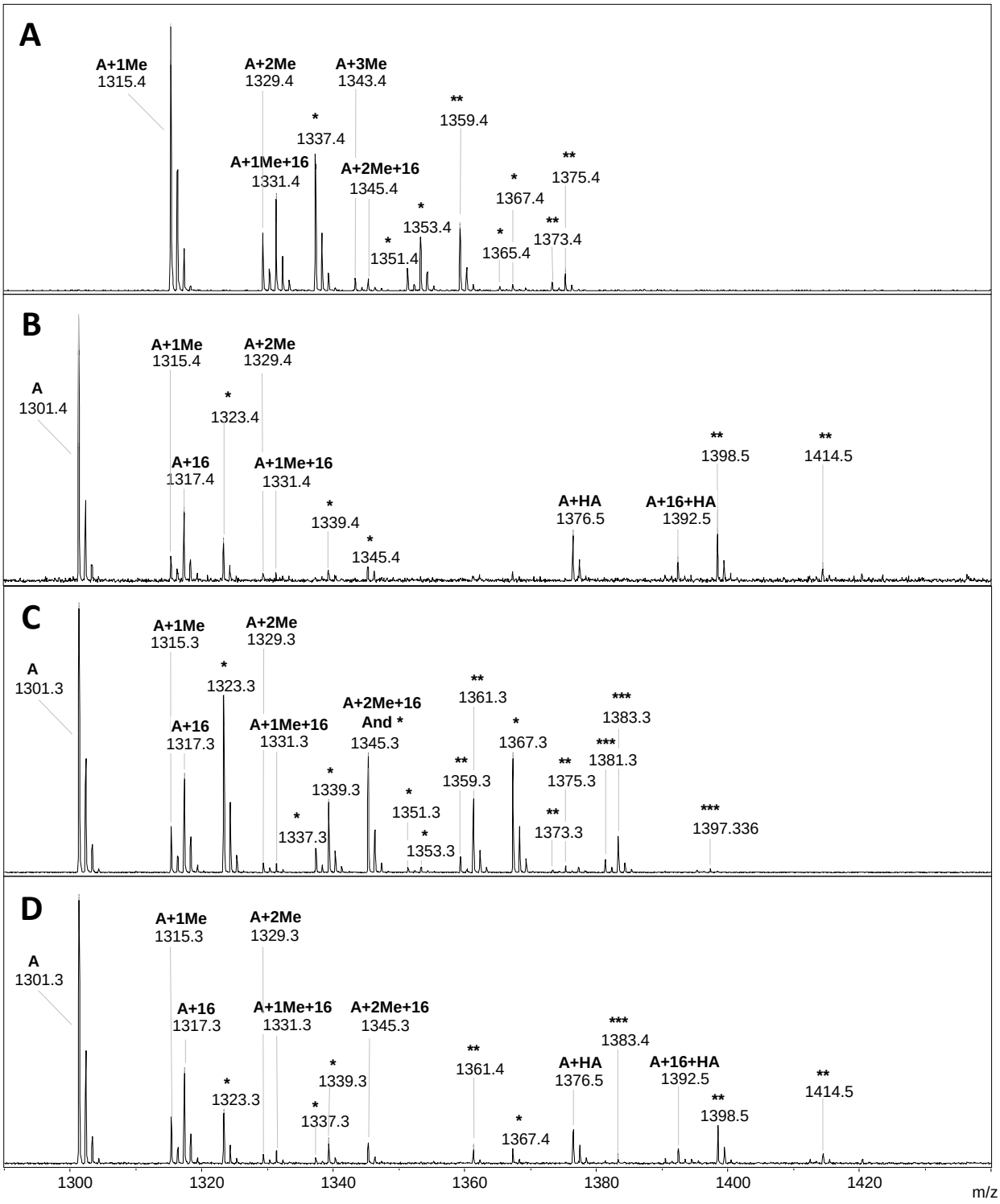


Figure II.6: MALDI-TOF mass spectra (positive mode). (A) Pool Bb recovered from RGII hydrolysate after AEC of 0.48 M TFA for 16 h at 40°C. (B) Pool Bd incubated with hepthylamine. (C) Pool Bc recovered from RGII hydrolysate after AEC of 0.48 M TFA for 16 h at 40°C. (D) Pool Bc incubated with hepthylamine. * (M+Na+); ** (M+2Na+); * (M+2Na+).**

II.3.d Backbone and short side chains (C, D, E)

Three short side chains, the dimeric C and D chains and the monomeric chain E, are present on the RGII backbone. Side chains C and D are difficult to extract, due to the acid-lability of ulosonic acid residues, and identifying their (low molecular weight) signals within the matrix peaks (Stevenson *et al.*, 1988; Séveno *et al.*, 2009). Molecule protection through $NaBH_4$ reduction (Stevenson *et al.*, 1988) or derivatisation with a fluorescent tag (Séveno *et al.*, 2009) have been used for detection. Side chains C and D are likely to be co-eluted with side chain B in Pool B and Ba as previously observed (Thomas *et al.*, 1989). In the present study these two dimers are expected to have been lost during the desalting steps. Side chain E, consisting of one α-L-Ara*f* residue linked *O*-3 to a Gal*p*A residue in the backbone is not always detected. Total or partial removal of this residue can be due to the action of pectolytic enzymes used for RGII extraction and to the TFA hydrolysis conditions used for side chains debranching (Pabst *et al.*, 2013).

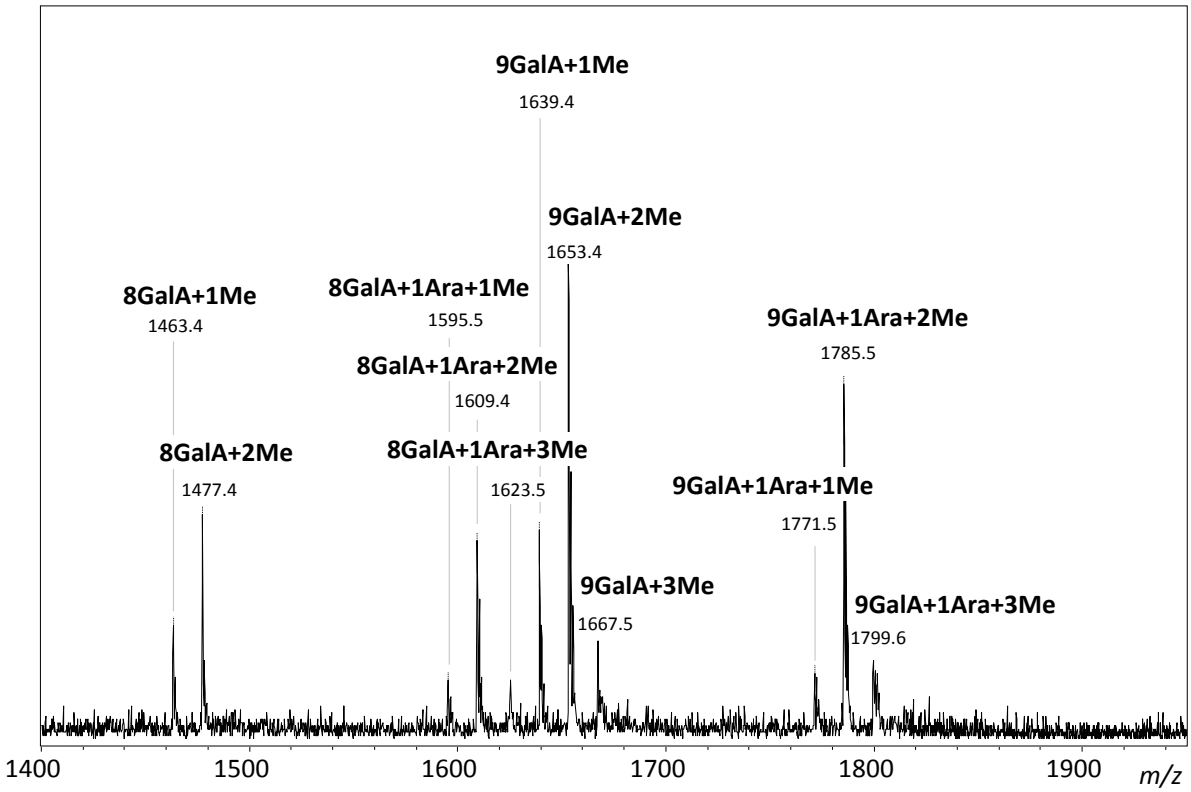


Figure II.7: MALDI-TOF mass spectrum of Pool Bd hydrolyzed by 0.1 M TFA for 8 h at 60°C. Zoom of the *m/z* 1400 – 1850 region. The full spectrum is shown on Supplementary Figure.I.1B.

Based on glycosyl residues molar composition and molar mass, a backbone's length of 6 to 11 residues has been estimated by Yapo (2011). DP values between 8 and 9 are the most commonly found structures (Melton *et al.*, 1986; Whitcombe *et al.*, 1995; Pellerin *et al.*, 1996). Higher DP values have been reported in sycamore (Whitcombe *et al.*, 1995) and in wine (Pellerin *et al.*, 1996). As RGII is covalently linked (1→4) to α-D-Gal*p*A HG (Ishii and Matsunaga, 2001), the boundary between HG and RGII is difficult to determine. Depending on extraction conditions, the presence of HG at the ends of RGII molecules cannot be precluded (Yapo, 2011). Conversely, oligogalacturonates of low DP (<6), which are likely to be due to partial acid degradation of the RGII backbone (Yapo, 2011), have been observed. The Gal*p*A residues can be methyl-esterified, as first reported by Melton *et al.* (1994).

In order to gain insight into (i) backbone length, (ii) presence of side chain E, and (iii) methyl-esterification status, different hydrolysis conditions ranging from 0.1 M TFA, 4 h, 60°C to 0.1 M TFA, 4 h, 80°C were applied to Pool Bd (Figure.II.2). The different hydrolysates were analysed by mass spectrometry (Supplemental Figure.II.1). No signal was detected at *m/z* < 1155. After 0.1 M TFA, 4 h, 60°C, matrix-related signals only were detected (Supplemental Figure.II.1A). After 0.1 M TFA, 8 h, 60 °C, several signals were observed that were assigned to different forms of side chain A (non-methylated, methylated and oxidized at *m/z* 1301, 1315 and 1317, respectively) (Supplemental Figure.II.1B) and oligogalacturonates of DP 8 and 9 bearing methyl groups from zero to three and/or an β-L-Ara*f* residue (Supplemental Figure.II.1B, Figure.II.7). The presence of di- and tri-methylated oligogalacturonates of DP 7 that exhibit the same molar mass than non-methylated and mono-methylated side chain A, respectively, cannot be precluded. After 0.1 M TFA, 16 h, 60°C (Supplemental Figure.II.1C) the spectrum obtained was very similar to the data presented in Supplemental Figure.II.1B, except for the appearance of a signal at *m/z* 1155, which can be attributed to a truncated form of side chain A (side chain A lacking 2-*O*-Me-α-D-Xyl). Interestingly, after hydrolysis at 80°C (Supplemental Figure.II.1D, E and F), signals corresponding to oligogalacturonates bearing one β-L-Ara*f* residue could no longer be detected. Furthermore, after the 80°C treatment the signals corresponding to 'full length' side chain A decreased in favour of signals corresponding to the truncated form of the oligosaccharide mentioned above.

II.4 Discussion

In the present study, different mild acid treatments were used to deconstruct wine RGII. AEC allowed good separation of side chain B, methyl-esterified and non-methyl-esterified side chain A. Hydrolysis conditions were shown to have a significant impact on the structures observed.

After hydrolysis of wine RGII with 0.1 M TFA at 40°C for 16 h, the main component released was side chain B with a DP of 9 that was mono-acetylated on the 2-*O*-Me-α-D-Fuc*p* residue. Trace amounts of non- and di-acetylated side chain B and mono-acetylated side chain B of DP 7 or 8 were also detected. The use of harsher hydrolysis conditions induced partial degradation of side chain B. β-L-Ara*f*, 2-*O*-Me-α-L-Fuc*p* and acetyl groups appeared particularly acid-labile. In a recent study, side chain B of DP 8, having lost its terminal β-L-Ara*f* residue, was claimed to be predominant in wine RGII (Pabst *et al.*, 2013). It is likely that in the latter study, the terminal β-L-Ara*f* residue was lost due to the harsh hydrolysis condition used. The present study provides evidence that the rhamnosylation status of side chain B is not highly affected by hydrolysis conditions. In *Arabidopsis thaliana* RGII, which lacks a terminal β-L-Ara*f* residue, rhamnosylation was shown to vary according to organ and developmental stage (Pabst *et al.*, 2013). From 2 to 18 days after imbibition single and double rhamnosylation of side chain B decreases, while a variant of the oligosaccharide lacking any α-L-Rha increases. In adult plants, rhamnosylation of side chain B appears organ-specific; the oligosaccharide decoration lacking α-L-Rha is the predominant structure in stems and siliques, while in leaves chain B generally contains a single α-L-Rha residue (Pabst *et al.*, 2013). The hydrophobic character of side chain B, due to the presence of Rha*p*, Ara*p*, *O*-Me and *O*-Ac groups, has been pointed out by Spellman *et al*. (1983). Variability in acetylation and/or in the length of side chain B could be a way to modulate the hydrophobic character of RGII and hence its association with other molecules through hydrophobic interactions. Arabinogalactan proteins, RGII and boron are co-located close to the plasma membrane (Matoh *et al*., 1998; González-Fontes *et al.*, 2008) and a boron-deficiency was shown to prevent the covalent linkage of a hydroxyproline-/proline-rich protein to bean (*Phaseolus vulgaris L.*) root nodules (Bonilla *et al.*, 1997). More recently, an arabinogalactan

protein-extensin (AGPE) from a legume nodule was extracted together with RGII using the MAC265 monoclonal antibody that binds to the glycoprotein. Dimeric RGII was shown to bind to the glycoprotein (Reguera *et al.*, 2010).

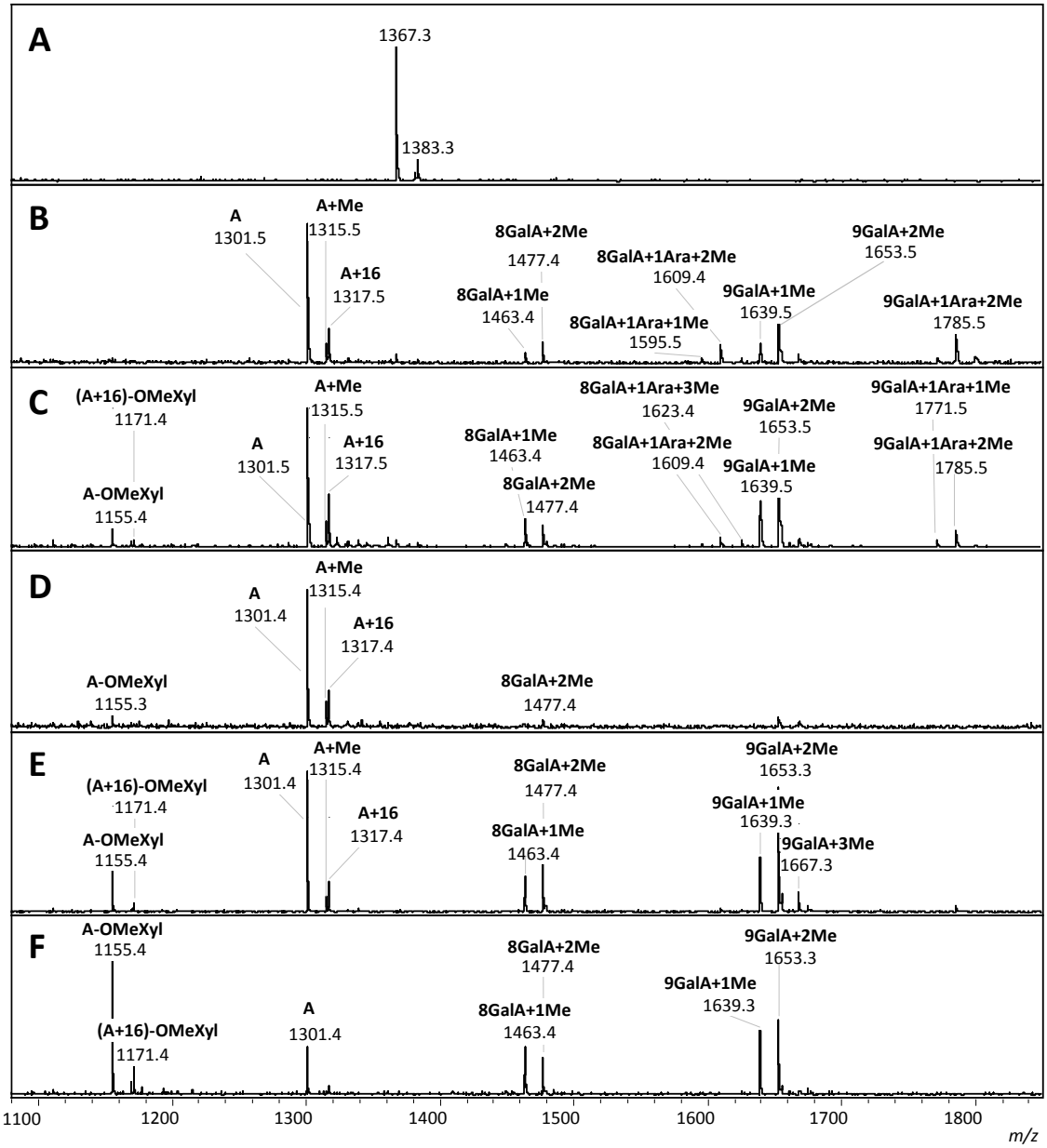
After hydrolysis of wine RGII with 0.48 M TFA at 40°C for 16 h, two forms of 'full length' side chain A, a methyl-esterified one and a non-methyl-esterified one, were recovered and separated by AEC. These two forms of 'full length' side chain A could also be recovered after hydrolysis with 0.1 M TFA at 60°C for 8 h. The use of harsher extraction conditions rapidly led to the appearance of a truncated form of side chain A having lost the *O*-Me-α-D-Xyl*p* residue. Analysis by mass spectrometry revealed that side chain A is structurally highly variable. Beside single methyl-esterification, single and double methyl-etherification and substitution of the central α-L-Fuc*p* residue by α-L-Gal*p* were evident, in good agreement with Pabst *et al.* (2013). In the present study, based on the relative intensities of MS signals, the proportion of chain A with substitution of α-L-Fuc*p* by α-L-Gal*p* is 27% is also in line with Pabst *et al*. 2013. This naturally occurring substitution provides an explanation for the detection of 3,4-linked-Gal*p* residues in purified wine RGII fractions, (Pellerin *et al.*, 1996). Pabst *et al.* (2013) screened for oxidized side chain A in different plants and showed it could constituted up to 45% of total side chain A. The natural presence of this modified form of side chain A in several plants allowed Pabst *et al.* (2013) to shed new light on several RGII mutant phenotypes. Indeed, substitution of α-L-Fuc*p* by α-L-Gal*p* had been previously described in the *Arabidopsis thaliana mur1* mutant, which lacked the gene encoding GDP-D-Man-4,6-dehydratase required for the conversion of GDP-D-Man to GDP-D-Fuc (Bonin *et al.*, 1997; O'Neill *et al.*, 2001; Reuhs *et al.*, 2004). The *mur1* mutant showed a decrease in RGII dimerization, which severely affected plant growth (O'Neill *et al.*, 2001). In this mutant side chain A was truncated upstream of the central β-L-Rha*p*, which led Pabst *et al.* (2013) to claim that the impact of the mutation on RGII dimerization could be due to truncation of side chain A rather than to the α-L-Fuc*p* substitution by α-L-Gal*p*. Silencing of GDP-D-Man-3,5-epimerase in tomato plants also resulted in a lower capacity of RGII to perform *in muro* cross-linking (Gilbert *et al.*, 2009; Voxeur *et al.*, 2011). The oxidised form of side chain A - encompassing an internal α-L-Gal*p* - represents 25% of total side chain A in *Solanum lycopersicum*. It was thereby hypothesized that truncation of side chain A - due to absence of

this internal α-L-Gal*p* - rather than a decrease in terminal α-L-Gal*p* content could be responsible for the observed phenotypes (Pabst *et al.*, 2013).

After hydrolysis of wine RGII with 0.1 M TFA at 60°C for 8 h or 16 h, backbone oligogalacturonates of DP 8 and 9 bearing zero or one β-L-Ara*f* residue were released. The use of harsher extraction conditions led to the loss of arabinosylated oligogalacturonates. Methyl-esterification of Gal*p*A residues in the backbone was also evident. Up to two methyl groups were detected on oligogalacturonates of DP 8 and up to three on oligogalacturonates of DP 9. Beside backbone methyl-esterification, the possible single methyl-esterification and single or double methyl-etherification of uronic acids in side chain A, recently shown by Pabst *et al.* (2013), was confirmed in the present study. Calcium was shown to reinforce RGII dimerization initiated by boron; calcium release promotes hydrolysis of RGII dimers (Fleischer *et al.*, 1999; Kobayashi *et al.*, 1999). Variability in the methylation status of side chain A and backbone could affect RGII dimer stabilisation with calcium.

RGII is a very complex structure that is overall well-conserved over plant evolution although some structural differences were observed from one land plant to another (Matsunaga *et al.*, 2004). In the present study, several modifications to wine RGII structure were identified. Some of them, such as dearabinosylation and deacetylation, were the consequence of acid treatment. Others, such as methyl-esterification, methyl-etherification and oxidation reflect a natural diversity. A range of RGII structures exhibiting specific physico-chemical properties such as hydrophobicity and charge density were shown to co-exist (this work; Pabst *et al.*, 2013) and to be organ-specific and developmentally regulated in *Arabidopsis thaliana* (Pabst *et al.*, 2013). The physiological significance of this variability remains, however, to be investigated.

Supplementary data



Supplemental Figure II.1: MALDI-TOF mass spectra of Pool Bd hydrolyzed by 0.1 M TFA: (A) for 4 h at 60 °C; (B) for 8 h at 60 °C; (C) for 16 h at 60°C; (D) for 1 h at 80 °C; (E) for 2 h at 80 °C; (F) for 4 h at 80 °C

References

Bonilla I, Mergold-Villasenor C, Campos ME, Sanchez N, Perez H, Lopez L, Castrejon L, Sanchez F, Cassab GI. 1997. The aberrant cell walls of boron-deficient bean root nodules have no covalently bound hydroxyproline-/proline-rich proteins. *Plant Physiology* **115**(4): 1329-1340

Bonin CP, Potter I, Vanzin GF, Reiter WD. 1997. The *MUR1* gene of Arabidopsis thaliana encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de *novo* synthesis of GDP-L-fucose. *Proceedings of the National Academy of Sciences of the United States of America* **94**(5): 2085-2090.

Caffall KH, Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate research* **344**(14): 1879-1900.

Darvill AG, McNeil M, Albersheim P. 1978. Structure of plant cell walls: VIII. A new pectic polysaccharide. *Plant Physiology* **62**(3): 418-422.

Doco T, Brillouet J-M. 1993. Isolation and characterisation of a rhamnogalacturonan II from red wine. *Carbohydrate Research* **243**(2): 333-343.

Doco T, Williams P, Vidal S, Pellerin P. 1997. Rhamnogalacturonan II, a dominant polysaccharide in juices produced by enzymic liquefaction of fruits and vegetables. *Carbohydrate research* **297**(2): 181-186.

Doco T, O'Neill MA, Pellerin P. 2001. Determination of the neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers* **46**(3): 249-259.

Edashige Y, Ishii T. 1998. Rhamnogalacturonan II from cell walls of *Cryptomeria japonica*. *Phytochemistry* **49**(3): 681-690.

Fleischer A, O'Neill MA, Ehwald R. 1999. The pore size of non-graminaceous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan II. *Plant Physiology* **121**(3): 829-838.

Gilbert L, Alhagdow M, Nunes-Nesi A, Quemener B, Guillon F, Bouchet B, Faurobert M, Gouble B, Page D, Garcia V, Petit J, Stevens R, Causse M, Fernie AR, Lahaye M, Rothan C, Baldet P 2009. GDP-d-mannose 3,5-epimerase (GME) plays a key role at the intersection of ascorbate and non-cellulosic cell-wall biosynthesis in tomato. *The Plant Journal* **60**(3): 499-508.

Glushka JN, Terrell M, York WS, O'Neill MA, Gucwa A, Darvill AG, Albersheim P, Prestegard JH. 2003. Primary structure of the 2-O-methyl-α-L-fucose-containing side chain of the pectic polysaccharide, rhamnogalacturonan II. *Carbohydrate research* **338**(4): 341-352.

González-Fontes A, Navarro-Gochicoa MT, Camacho-Cristóbal JJ, Herrera-Rodríguez MB, Quiles-Pando C, Rexach J. 2008. Is Ca2+ involved in the signal transduction pathway of boron deficiency? New hypotheses for sensing boron deprivation. *Plant Science* **217-218**: 135-139

Ishii T, Ono H. 1999. NMR spectroscopic analysis of the borate diol esters of methyl apiofuranosides. *Carbohydrate Research* **321**(3-4): 257-260.

Ishii T, Matsunaga T. 2001. Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry* **57**(6): 969-974.

Ishii T, Matsunaga T, Hayashi N. 2001. Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiology* **126**(4): 1698-1705.

Kaneko S, Ishii T, Matsunaga T. 1997. A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochemistry* **44**(2): 243-248.

Kobayashi M, Matoh T, Azuma J. 1996. Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. *Plant Physiology* **110**(3): 1017-1020.

Kobayashi M, Ohno K, Matoh T. 1997. Boron nutrition of cultured tobacco BY-2 Cells. II. Characterization of the boron-polysaccharide complex. *Plant & Cell Physiology* **38**(6): 676-683.

Kobayashi M, Nakagawa H, Asaka T, Matoh T. 1999. Borate-rhamnogalacturonan II bonding reinforced by Ca^{2+} retains pectic polysaccharides in higher-plant cell walls. *Plant Physiology* **119**(1): 199-204.

Matoh T, Kawaguchi S, Kobayashi M. 1996. Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls of higher plants. *Plant & cell physiology* **37**(5): 636-640.

Matoh T, Kobayashi M. 1998. Boron and calcium, essential inorganic constituents of pectic polysaccharides in higher plant cell walls. *Journal of Plant Research* **111**(1): 179-190.

Matoh T, Takasaki M, Takabe K, Kobayashi M. 1998. Immunocytochemistry of rhamnogalacturonan II in cell walls of higher plants. *Plant and Cell Physiology* **5**: 9.

Matoh T, Takasaki M, Kobayashi M, Takabe K. 2000. Boron nutrition of cultured tobacco BY-2 cells. III. Characterization of the boron-rhamnogalacturonan II complex in cells acclimated to low levels of boron. *Plant & cell physiology* **41**(3): 363-666.

Matsunaga T, Ishii T, Matsumoto S, Higuchi M, Darvill A, Albersheim P, O'Neill MA. 2004. Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. Implications for the evolution of vascular plants. *Plant physiology* **134**(1): 339-351.

Melton LD, McNeil M, Darvill AG, Albersheim P, Dell A. 1986. Structural characterization of oligosaccharides isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research* **146**(2): 279-305.

Mohnen D. 2008. Pectin structure and biosynthesis. *Current Opinion in Plant Biology* **11**(3): 266-277.

O'Neill MA, Albersheim P, Darvill AG. 1990. The pectic polysaccharides of primary cell walls.In: e. P. Dey. *Methods in plant biochemistry* Academic Press, London, UK, 2: 415-441

O'Neill MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill AG, Albersheim P. 1996. Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. *The Journal of biological chemistry* **271**(37): 22923-22930.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG. 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* **294**: 846-849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual review of plant biology* **55**: 109-139.

Pabst M, Fischl RM, Brecker L, Morelle W, Fauland A, Kofeler H, Altmann F, Leonard R. 2013. Rhamnogalacturonan II structure shows variation in the side chains

monosaccharide composition and methylation status within and across different plant species. *Plant journal for cell and molecular biology* **76**(1): 61-72.

Pellerin P, Doco T, Vidal S, Williams P, Brillouet J-M, O'Neill MA. 1996. Structural characterization of red wine rhamnogalacturonan II. *Carbohydrate Research* **290**(2): 183-197.

Pérez S, Rodríguez-Carvajal MA, Doco T. 2003. A complex plant cell wall polysaccharide: rhamnogalacturonan II. A structure in quest of a function. *Biochimie* **85**(1-2): 109-121.

Puvanesarajah V, Darvill AG, Albersheim P. 1991. Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. *Carbohydrate Research* **218**: 211-222.

Reguera M, Abreu I, Brewin NJ, Bonilla I, Bolanos L. 2010. Borate promotes the formation of a complex between legume AGP-extensin and Rhamnogalacturonan II and enhances production of Rhizobium capsular polysaccharide during infection thread development in Pisum sativum symbiotic root nodules. *Plant, Cell & Environment* **33**(12): 2112-2120.

Reuhs BL, Glenn J, Stephens SB, Kim JS, Christie DB, Glushka JG, Zablackis E, Albersheim P, Darvill AG, O'Neill MA. 2004. L-Galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient *mur1 Arabidopsis* mutant. *Planta* **219**(1): 147-157.

Ridley BL, O'Neill MA, Mohnen DA. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**(6): 929-967.

Rodríguez-Carvajal MA, Hervé du Penhoat C, Mazeau K, Doco T, Pérez S. 2003. The three-dimensional structure of the mega-oligosaccharide rhamnogalacturonan II monomer: a combined molecular modeling and NMR investigation. *Carbohydrate Research* **338**(7): 651-671.

Ropartz D, Bodet PE, Przybylski C, Gonnet F, Daniel R, Fer M, Helbert W, Bertrand D, Rogniaux H. 2011. Performance evaluation on a wide set of matrix-assisted laser desorption ionization matrices for the detection of oligosaccharides in a high-throughput mass spectrometric screening of carbohydrate depolymerizing enzymes. *Rapid communications in mass spectrometry* **25**(14): 2059-2070.

Séveno M, Voxeur A, Rihouey C, Wu AM, Ishii T, Chevalier C, Ralet MC, Driouich A, Marchant A, Lerouge P. 2009. Structural characterisation of the pectic polysaccharide rhamnogalacturonan II using an acidic fingerprinting methodology. *Planta* **230**(5): 947-957.

Shimokawa T, Ishii T, Matsunaga T. 1999. Isolation and structural characterization of rhamnogalacturonan II-borate complex from Pinus densiflora. *Journal of Wood Science* **45**(5): 435-439.

Shin K-S, Kiyohara H, Matsumoto T, Yamada H. 1998. Rhamnogalacturonan II dimers cross-linked by borate diesters from the leaves of *Panax ginseng* C.A. Meyer are responsible for expression of their IL-6 production enhancing activities. *Carbohydrate Research* **307**(1-2): 97-106.

Spellman MW, McNeil M, Darvill AG, Albersheim P, Dell A. 1983. Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research* **122**(1): 131-153.

Stevenson TT, Darvill AG, Albersheim P. 1988. Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydrate Research* **182**(2): 207-226.

Strasser GR, Amadò R. 2001. Pectic substances from red beet (*Beta vulgaris conditiva*). Part I. Structural analysis of rhamnogalacturonan I using enzymic degradation and methylation analysis. *Carbohydrate Polymers* **44**(1): 63-70.

Thibault JF. 1979. Automatisation du dosage des substances pectiques par la methode au méta-hydroxydiphényle. *Lebensmittel-Wissenschaft und-Technologie* **71**: 215-223.

Thomas JR, Darvill AG, Albersheim P. 1989. Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Carbohydrate Research* **185**(2): 261-277.

Tollier MT, Robin JP. 1979. Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Annales de Technologie Agricole* **28**: 1-15.

Vidal S, Salmon J-M, Williams P, Pellerin P. 1999. *Penicillium daleae*, a soil fungus able to degrade rhamnogalacturonan II, a complex pectic polysaccharide. *Enzyme and Microbial Technology* **24**(5-6): 283-290.

Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA, Glushka J, Darvill AG, Albersheim P. 2000. Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydrate research* **326**(4): 277-294.

Vidal S, Williams P, Doco T, Moutounet M, Pellerin P. 2003. The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers* **54**(4): 439-447.

Voxeur A, Gilbert L, Rihouey C, Driouich A, Rothan C, Baldet P, Lerouge P. 2011. Silencing of the GDP-D-mannose 3,5-epimerase affects the structure and cross-linking of the pectic polysaccharide rhamnogalacturonan II and plant growth in tomato. *The Journal of biological chemistry* **286**(10): 8014-8020.

Whitcombe AJ, O'Neill MA, Steffan W, Albersheim P, Darvill AG. 1995. Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II. *Carbohydrate Research* **271**(1): 15-29.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001. Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology* **47**(1): 9-27.

Yapo BM, Lerouge P, Thibault J-F, Ralet M-C. 2007. Pectins from citrus peel cell walls contain homogalacturonans homogenous with respect to molar mass, rhamnogalacturonan I and rhamnogalacturonan II. *Carbohydrate Polymers* **69**(3): 426-435.

Yapo BM. 2011. Pectin Rhamnogalacturonan II: On the "Small Stem with Four Branches" in the Primary Cell Walls of Plants. *International Journal of Carbohydrate Chemistry* **2011**: 1-11.

York WS, Darvill AG, McNeil M, Albersheim P. 1985. 3-deoxy-d-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. *Carbohydrate Research* **138**(1): 109-126.

CHAPTER III

New monoclonal antibodies, which recognize an atypical arabinogalactan motif

III.1. Introduction

Pectin is a major constituent of primary cell walls in eudicotyledonous plants. Numerous functions of pectin in plants have been described including cell signaling (Branca *et al.*, 1988; Marfà *et al.*, 1991; Shibuya and Minami, 2001), cell-to-cell adhesion (O'Neill *et al.*, 2001; Willats *et al.*, 2001a), cell wall hydration (Tang *et al.*, 1999; Ha *et al.*, 2005; Larsen *et al.*) and cell expansion (Proseus and Boyer, 2006). It has also been suggested that pectin could act as a storage polysaccharide (Buckeridge *et al.*, 2005; Gomez *et al.*, 2009). However functions *in planta* related to specific structures remain unclear. Pectin is a multibloc polymer composed of 3 distinct domains, which are covalently linked to each other (Ishii and Matsunaga, 2001; Caffall and Mohnen, 2009): homogalacturonan (HG), HG analogues and rhamnogalacturonan I (RGI). HG is a linear structure made of (1→4) linked α-D-Gal*p*A units that can be methyl esterified at C-6 and acetyl esterified at *O*-2 or *O*-3 (Ridley *et al.*, 2001). HG analogues consist in a α-D-Gal*p*A backbone substituted at *O*-2 and/or *O*-3 with monomers or short side chains. Homogalacturonan substituted with Xyl*p* is called xylogalacturonan while homogalacturonan

substituted with Api*f* gives rise to apiogalacturonan (Ovodov *et al.*, 1971; Le Goff *et al.*, 2001). Rhamnogalacturonan II is decorated with five different side chains (A-E) composed of specific and peculiar sugars, branched on the α-D-Gal*p*A backbone (O'Neill *et al.*, 2004). RGI is the only pectic domain with a heterobackbone having a repeating dimer sequence of [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]. Uronic acid from RGI can be acetylated at the *O*-2 and *O*-3 position. 20-80 % of the Rha*p* residues can be substituted at *O*-4 and, to a lower extent, at *O*-3 with neutral side chains (Albersheim *et al.*, 1996; Ridley *et al.*, 2001). RGI side chains include galactan, arabinan or type I (AGI) and II (AGII) arabinogalactan (Carpita and Gibeaut, 1993; Willats *et al.*, 2001a; Caffall and Mohnen, 2009). Over 15 different linkages were described in the RGI side chains (Lau *et al.*, 1987; Caffall and Mohnen, 2009). Arabinan consists of a (1→5)-α-L-Ara*f* backbone, which can be branched at *O*-3 with Ara*f* units or/and arabinan secondary side chains. Galactan and AGI are both made of a (1→4)-β-D-Gal*p* backbone on which substitutions by monomeric Gal*p* units at the *O*-6 or at the *O*-3 position can occur. AGI is substituted with α-L-Ara*f*-*p* residues and/or with (1→5)-α-L-Ara*f* short side chains. AGII is composed of a (1→3)-β-D-Gal*p* backbone decorated with (1→6)-β-D-Gal*p* secondary chains, which are arabinosylated. According to plant sources, organs, tissues and developmental stages, RGI side chains composition, length and degree of branching are very variable (Lerouge *et al.*, 1993; Willats *et al.*, 2001a; Caffall and Mohnen, 2009).

To explore the variability of polysaccharide domains in plant cell walls, different methods have been used. Without the need to stain or label sample, chemical mapping using Fourier Transform Infra Red (FT-IR) and Raman spectroscopy can provide information on chemical composition of tissues down to the level of individual cells. These techniques have been used to study pectin in various plants and organs (Mouille *et al.*, 2003; Jones *et al.*, 2005; Mazurek *et al.*, 2013; Chylinska *et al.*, 2014). However the sensitivity of these methods is limited and spectra are not always straightforward to interpret. Therefore, high resolution imaging with the aid of specific monoclonal antibodies (mAbs) has been the method of choice to identify different polysaccharidic domains within cell walls *in planta* (Willats *et al.*, 2001a). A large set of mAbs has already been developed against pectin motifs. Available mAbs can recognize different methylation patterns of HG (LM7 -Willats *et al.*, 2001- ; LM18 -Verhertbruggen *et al.*, 2009b- ; LM19 -Verhertbruggen *et al.*, 2009b- ; LM20 -Verhertbruggen *et al.*, 2009b- ; JIM7 -Knox *et al*., 1990- ; JIM5 Knox *et al*., 1990-). LM8 is a mAb specific to

xylogalacturonan (Willats *et al.*, 2004). Various mAbs have also been generated against RGI. The unbranched backbone is recognized by INRA-RU1, INRA-RU2 and CCR1 (Young *et al.*, 2008; Ralet *et al.*, 2010). LM5 (Jones *et al.*, 1997) and LM9 (Clausen *et al.*, 2003) are specific to unsubstituted and feruloylated linear galactans, respectively. Three mAbs directed to arabinans can detect different features of arabinan structures (LM6 -Willats *et al.*, 1998- ; LM13 -Verhertbruggen *et al.*, 2009a- ; LM16 -Verhertbruggen *et al.*, 2009a-). Most of the available mAbs to RGI bind to linear pectic structures. Antibodies directed to highly-branched pectic domains or to the connection between the side chains and the RGI backbone are still missing. Here, we describe the production and characterization of a novel antibody that binds to a structure, which belongs to RGI side chains. The epitope is detected in galactan-rich plant sources and is expected to have an essential function in the wall. This new antibody will extend the panel of probes available to label pectin domains in the context of intact cells.

III.2. Materials and methods

III.2.a. Plant material, enzymes and glycans

Plant material

Potato pulp (Ref: 74739; 2003) was provided by Roquette (Lestrem, France).

Enzymes

α-amylase (Termamyl 120 L, 120 KNU/g, Novozyme) and amyloglucosidase (Aspergillus *niger*, A.3042, 6000 U/mL, Sigma) were used for destarching. The 1,4-β-galactanase (940 UI/mL; from *Aspergillus niger*) was obtained from Megazyme (Bray, Ireland). 1,5-α-arabinanase (950 UI/mL) was prepared as previously reported (Bonnin *et al.*, 2002). Rhamnogalacturonan hydrolase (from *Aspergillus aculeatus*; Batch PPJ4478) is from Novozyme (Copenhagen, Denmark).

Glycans

Polysaccharides used in the present work are referenced in Table.III.1.

Table III.1: Sources and sugar composition of polysaccharides used for the characterization of mAb supernatants

Glycans	Sources	Sugar composition (%)
Sugar beet pectin (SBP6230)	Ralet, *et al*., 2003	Rha:6.4; Ara:14.9; Gal:12.6; GalA:66.2
Lemon pectin	Moller, *et al*., 2008	ND
Apple pectin	Moller, *et al*., 2008	ND
Lime pectin DM: 81% (E81)	Ralet, *et al*., 2001	Rha:1.5; Ara:0.3; Gal:4.7; GalA:93.5
Lime pectin DM: 15% (B15)	Ralet, *et al*., 2001	Rha:0.9; Ara:0.2; Gal:1.8; GalA:97.1
Lime pectin DM: 34% (B34)	Ralet, *et al*., 2001	Rha:0.9; Ara:0.2; Gal:1.8; GalA:97.1
HG citrus (DM 0)	Tanhatan-Nasseri *et al*., 2010	GalA > 98%
RGI soybean	Megazyme	Rha:13; Ara: 3.5; Gal:12.5; GalA:51; Fuc:10 ;Xyl: 13.7
RGI beetroot	INRA-Nantes	Rha:7; Ara: 47; Gal:10; GalA:36
RGI carrot	INRA-Nantes	Rha:5.3; Ara:6; Gal:23.1; GalA:62.5
AG II (Larch wood)	Sigma	ND
AGP (Larch wood)	Megazyme	Purty > 85% (Ara: 15 ; Gal: 85)
Potato pectic galactan	Megazyme	Rha:3; Ara:6; Gal:82; GalA:9
Potato galactan I	Megazyme	Rha:3; Ara:2; Gal: 88; GalA:7
Potato galactan II	Lahaye *et al*., 1991	Rha:0.2; Ara:1.6; Gal:86.4; GalA:1.4
Lupin galactan	Megazyme	Rha:4.2; Ara:20.9; Gal:71.3; GalA:5
Branched arabinan (sugar beet)	Megazyme	Purity 95% (Rha: 2; Ara: 88; Gal: 3; GalA:7)
Linear arabinan (sugar beet)	Megazyme	Ara: 97.5; Gal:0.4; Rha:0.1; GalA:2

RGI backbone oligosaccharides (designated RU-oligosaccharides; R:Rha and U:GalA) refer to a pool encompassing a mixture of R_nU_n oligosaccharides (n:2-18) prepared from *A. thaliana* seed mucilage as described in Ralet *et al.,* (2010). Briefly, water-soluble mucilage was hydrolyzed by a rhamnogalaturonan hydrolase for 3 h. The digest was precipitated by ethanol, and the ethanol soluble fraction was recovered, desalted and freeze-dried. DP-resolved RGI oligosaccharides (designated RxUy(Gz), R: Rha; U:GalA; G:Gal) were produced from *A. thaliana* seed mucilage or sugar beet pulp as described in Ralet *et al.,* (2010). Arabinan oligosaccharides (designated Ara-oligosaccharides) refer to a pool encompassing a

mixture of (1→5)-α-L-Ara*f* oligosaccharides (DP5-12) (Ralet *et al.,* 2010). DP-resolved Ara-oligosaccharides were further recovered using size-exclusion chromatography. Galactan oligosaccharides (designated Gal- oligosaccharides) refer to a pool encompassing a mixture of (1→4)-β-D-Gal*p* oligosaccharides (DP4-15) (Ralet *et al.,* 2010). DP-resolved Gal-oligosaccharides were further recovered using size-exclusion chromatography. Feruloylated Ara- and Gal-oligosaccharides were prepared according to Ralet *et al*., (1994).

III.2.b. Potato pectin extraction

The de-starched potato pulp was mixed with 0.1 M NaOH (3 L) at 90°C for 2 h. Potato pulp (Roquette, France) (*Solanum tuberosum*) (100 g) was de-starched using an α-amylase (Termamyl 120 L, Novozyme) (10 mL at 15U/mL) in 80 mM sodium phosphate buffer pH 6 (2 L) for 25 min at 90°C. The suspension was cooled down to 30°C and pH was brought to 4.5 by 1 M HCl. The suspension was then incubated with amyloglucosidase (*Aspergillus Niger*, A.3042 Sigma, 1 mL, 6000 U/mL) for 17 h at 60°C. Fresh amyloglucosidase (1 mL, 6000 U/mL) was added at time 30 min and 60 min. The mixture was then cooled and pH brought to pH 7.8 with 1 M NaOH. The de-starched potato pulp was mixed with 0.1 M NaOH (3 L) at 90°C for 2 h. These conditions favor the β-elimination of methylesterified HG, resulting in the degradation of part of the HG component and enrichment in pectic RGI. After filtration and NaOH neutralization with 1 M HCl, the solution was concentrated to 1.5 L by rotary in vacuum evaporation at 40°C and raw RGI was recovered by precipitation with 70% ethanol overnight at 4°C. The suspension was centrifuged and the pellet was recovered and dissolved in water. The solution was concentrated by evaporation to 1 L to eliminate alcohol traces. The whole protocol starting with ethanol precipitation was repeated twice. Finally, the residual salts present in the raw RGI solution were removed by dialysis. The salt free solution was filtrated on a 3 µm membrane and freeze-dried.

III.2.c. Tailored RGI purification

(This part was performed by Valerie Cornault from University of Leeds, UK)

The raw RGI fraction (1200 mg) was pre-purified in three batches using DEAE-Sepharose fast flow gel. After pre-equilibration using 20 mM sodium acetate pH 4.5, the sample (400 mg in 40 mL H_2O) was loaded onto the chromatography gel (150 mL). The matrix was manually stirred over 20 min to allow the polysaccharides to fix before the vacuum aspiration of the buffer. The sample was eluted using 4 x 100 mL of 20 mM sodium acetate buffer pH 4.5 followed by 4 x 100 mL of 50 mM acetate pH 4.5 + 0.6 M NaCl. An additional step of 100 mL of 50 mM sodium acetate pH 4.5 + 1 M NaCl was added to elute the most acidic polymers. The sample eluting with 20 mM sodium acetate pH 4.5 (designated purified RGI) was dialyzed and freeze-dried.

Purified RGI (650 mg/130 mL) was dissolved in 50 mM sodium acetate buffer pH 4.5 (10 mL). Solution was incubated with an endo-1,4-β-galactanase (47 UI/mL, i.e 9.4 U/mg substrate; 450 U/mg protein; protein/substrate 2/1000) for 150 min at 40 °C. Then, the solution was boiled for 10 min at 100 °C to inhibit the enzyme and dialyzed (8000-10000 Mw) against distilled water for 3 days at 4 °C. The recovered RGI with shorter galactan side chains (2 mg/mL) was treated with an endo-arabinanase (pure enzyme, i.e enzyme/substrate 1/87; V/V) to degrade linear arabinose side chains. The reaction was performed in 50 mM acetate buffer at pH 4.5, for 24 h at 30 °C. After enzyme inactivation by boiling (as describe above), the digest was dialyzed against distilled water and the retentate was concentrated and freeze-dried. This fraction was designated ‘low-branched RGI’. RGI backbone was then split using rhamnogalacturonan hydrolase (Ralet *et al.*, 2010). RGI (1 mg/mL) was dissolved in 10 mM Na-acetate buffer, pH 4 and enzyme (1 mg/mL, i.e enzyme/substrate 1/390; w/w) was added. The solution was incubated at 40°C for 180 min. One volume of ethanol was added to the hydrolyzate to precipitate fragments with high degree of polymerization. After one night at 4 °C, suspension was centrifuged and supernatant was recovered, concentrated and then desalted using size exclusion chromatography on Sephadex G10 (77 cm x 44 mm). The column was equilibrated with degassed deionized water. RGI oligosaccharides were loaded and eluted water at a flow rate of 0.4 mL/min, at room temperature. Fractions containing glycans (RUP oligosaccharides) were collected and freeze-dried. All the different steps followed for RUP oligosaccharides purification are summarized in Figure.III.1.

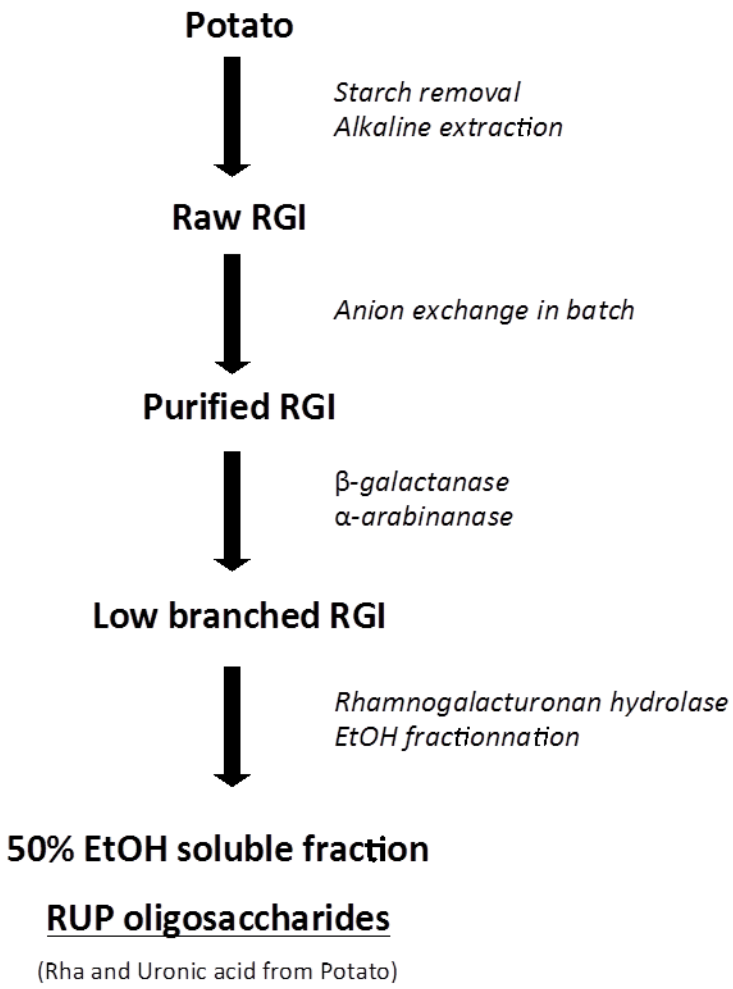


Figure III.1: Different steps followed for RUP oligosaccharides purification

III.2.d. Anion-exchange chromatography

RUP oligosaccharides were run through anion-exchange chromatography. Separation was performed at room temperature using a DEAE-Sepharose fast flow gel column (1.6 × 13.5 cm). The gel was equilibrated with degassed 25 mM Na-acetate buffer pH 5.5 at a flow rate of 1 mL/min. RUP oligosaccharides (20 mg/2 mL) were loaded onto the column. The gel was washed with 51.6 mL of 25 mM Na acetate buffer. Then, the bound material was eluted with a linear gradient of NaCl (0.025-0.2 M) in 25 mM Na acetate buffer (262 mL) and then with 0.5 M NaCl in 25 mM Na acetate buffer (34 mL). RUP oligosaccharides were recovered in 90 tubes that were analyzed for their neutral sugar and galacturonic acid contents.

III.2.e. Sugar analysis

Uronic acid and neutral sugars analyses

Uronic acid content was determined by the automated *m*-hydroxybiphenyl assay (Thibault, 1979). The difference in response of glucuronic acid and galacturonic acid in presence and absence of tetraborate was used to differentiate them (Renard *et al.*, 1999). Total neutral sugars were quantified by orcinol assay (Tollier and Robin, 1979) with correction for the galacturonic acid interference (Tollier and Robin, 1979). Individual neutral sugars were determined by generation of their alditol acetates after 150 min hydrolysis in 2 M trifluoroacetic acid at 120°C (Englyst and Cummings, 1988). Alditol acetates were analysed by GC (Perkin-Elmer autosystem). Samples were run on a fused-silica capillary column (30 m x 0.32 mm) bounded with OV-225 (50% cyanopropylphenyl dimethylpolysiloxane). Injection was carried at 210 °C with hydrogen (70 KPa) as a vector gas. Detection (FID) was performed at 220 °C.

Glycosidic linkage analyses

NaOH-DMSO reagent was prepared as described by Anumula and Taylor (1992). Acidic samples (1mg/mL) were converted into their H^+ form by percolating the solutions through a 1 mL Sigma Dowex® 50WX4 resin and freeze-dried. Then, samples were dried in a vacuum oven at 40°C for 2 h before being dissolved in dimethylsulfoxyde (DMSO, 0.2 mL) and sonicated for 2 min (Anumula and Taylor, 1992). Mixtures were left 30 min at ambient temperature before adding the NaOH-DMSO reagent (0.2 mL) (Anumula and Taylor, 1992). Methylation was performed with 0.1 mL of methyl iodide for 10 min. For reaction improvement, mixtures were sonicated and vortexed several times. Water (2 mL) was added and methylated products were extracted with chloroform (2 mL). Solutions were vigorously vortexed before brief centrifugation, which allows a strict separation of two phases. The aqueous supernatant phase was removed by aspiration. The organic phase was washed three times with water (2 mL) and dried under a stream of N_2. Methylated carbohydrates were hydrolyzed with 2 M trifluoroacetic acid and converted to their alditol acetates (see

previous paragraph). The partially methylated alditol acetates were analyzed by GC/MS (TRACE-GC-ISQ, Thermo™) on a non-polar thermo scientific™ TraceGOLD™ TG-1MS GC Column (30 m x 0.25 mm x 0.25µm). The carrier gas (hydrogen) was injected at 240 °C. The column oven temperature was maintained for 5 min at 60 °C and increased up to 315 °C (3 °C /min), where it was maintained for 2 min. The flow rate was set at 1.5 mL/min. The ion source temperature of the electron impact (EI) mass spectrometer was 230 °C. Masses were acquired with a scan range from *m/z* 100 to 500. Identification of partially methylated alditol acetates was based on their retention time and confirmed by GC-MS. Samples were also analysed on a GC Perkin-Elmer autosystem, on a fused-silica capillary column (30 m x 0.32 mm) bounded with OV-225 (50% cyanopropylphenyl dimethylpolysiloxane). Injection was carried at 220 °C with hydrogen (80 KPa) as a vector gas. Detection was performed at 220 °C with a flame ionization detector (FID). Both analyses results were averaged as results converged closely.

III.2.f. Neoglycoprotein preparation

Neoglycoproteins were prepared by coupling the RUP oligosaccharide pool by reductive amination to bovine serum albumin (BSA) for indirect ELISA and ovalbumin (OVA) for immunization (Roy *et al.*, 1984). Some arabino (dp 4-15) and galacto-oligosacharides (dp 5-12) produced by Ralet *et al*., (2010) were only conjugated to BSA. RU oligosaccharides coupled to BSA have been prepared by Ralet *et al*., (2010). Proteins (1.2mL of a 0.028 µmol/mL solution), oligosaccharides (0.2mL of a 0.017 µmol/mL solution) and $NaBH_3CN$ (42 mg/mL) were mixed in 0.1 M borate buffer (pH 9) and incubated 48 h at 45°C. Reaction was stopped by desalting conjugates on a PD-10 column (GE-Healthcare). The relative proportion of carbohydrate to protein in the conjugates was determined using the orcinol and *m*-hydroxybiphenyl methods for carbohydrates (Thibault, 1979; Tollier and Robin, 1979), and Bradford method (Bradford, 1976) for proteins. The weight ratio of proteins to oligosaccharides was in the range 0.60-0.75 for BSA conjugates and 0.34-0.43 for OVA conjugates (Table.III.2).

Table III.2: Oligosaccharide-protein conjugates (weight ratio of protein to oligosaccharides)

oligosaccharides	sugar/protein
Ara-BSA*	0.64
Gal-BSA*	0.75
RU-BSA**	0.73
RUP-BSA	0.7
RUP-OVA	0.43

Ara-BSA (arabino-oligosaccharides coupled to BSA); Gal-BSA (galacto-oligosaccharides coupled to BSA); RU-BSA (R_nU_n oligosaccharides coupled to BSA); RUP-BSA (RUP coupled with BSA); RUP-OVA (RUP coupled to OVA); *Oligosaccharides prepared by Ralet *et al.*, 2010; ** Oligosaccharides-protein conjugate prepared by Ralet *et al.*, 2010.

III.2.g. Production of antibodies

Two intrapodal injections at 10 days interval were performed in 3 mice. 30 µg of RUP-OVA were injected (about 15 µg/foot). Four days after the last injection, ganglionic lymphocytes were isolated and fused with myeloma NS1. Hybridoma were selected in Roswell Park Memorial Institute media (Lonza, RPMI 1640, BE-12-167F) supplemented with 1% non-essential amino acid (Lonza, NEAA 100X, BE-13-605E), 1% L-glutamine (Lonza, BE-13-605E), 1% penicillin-streptomycin (Lonza, 10000U penicillin/mL DE-17-602E), 5% hybridoma cloning supplement (PAA GmbH, F05- 009), 1X Hypoxanthine-aminopterin-thymidine (Sigma, supplement media 50X, hybrid-max) and 20% fetal calf serum (PAA clone, A15-102). Hybridomas were cloned by limited dilution. Hybridomas supernatants from the fusion were screened for the presence of anti-RUP oligosaccharides. They were tested in indirect ELISA and clones of interest were recloned by limited dilution. ELISA was performed using RUP-BSA and pure BSA to select clones that produce antibodies reacting only with carbohydrate moieties. For all the experiments, the crude hybridoma supernatants were used as a source of monoclonal antibodies.

III.2.h. Elisa experiments

Indirect ELISA

Plates were coated overnight at 4 °C with BSA or oligosaccharides coupled to BSA (5 µg per well) diluted in 10 mM carbonate-bicarbonate (Na_2CO_3 15 mM + $NaHCO_3$ 35 mM) buffer, pH 9.6. Uncoated sites were saturated with 200 µL of 4 % defatted skimmed milk in phosphate-buffered saline at pH 7.4 (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) for 1 h at room temperature. Hybridoma supernatants (50 µL) at appropriate dilution in 0.1 % defatted skimmed milk PBS (PBS0.1%) were added and incubated for 1 h at room temperature and followed by washing with PBS containing 0.05 % Tween 20. The bound antibodies were then incubated with (1/3000 diluted in PBS0.1%) anti-mouse IgG conjugated to horseradish peroxidase (170-6516, Bio-Rad, Carlsbad, CA, USA). After washing, color was revealed using orthophenylenediamine (Sigma, St Louis, MO, USA) as a substrate. Color reaction was stopped after 20 min with 50 µL of 2 M H_2SO_4 and the absorbance was read at 492 nm using an ELISA reader (Bio-Tek EL808).

ELISA competitive inhibition

Before performing ELISA competitive inhibition, all hybridoma supernatants were tested in indirect ELISA to evaluate the adequate concentration for an absorbance value of 1. ELISA competitive inhibition was used to check the reactivity of available antibodies towards RUP and to supernatants containing mAbs. To further investigate the specificity of the antibodies, they were pre-incubated with serial dilution of competitors in PBS containing 0.1% skimmed milk for 1 h at room temperature. The antibody-antigen mixtures (50/50 µL) were added to plates coated with BSA-RUP. All subsequent steps were performed as described above. The concentration of competitor giving a 50% inhibition (IC50) of the binding was determined by plotting the competitor concentration versus absorbance. Values from controls with no competitor were taken as 0% inhibition, and values from controls with no antibody represented 100% inhibition of binding.

ELISA competitive inhibition was also performed using, as a competitor RUP oligosaccharide fractions separated by ion exchange chromatography. Each fraction was fivefold diluted, with 25 mM Na-acetate buffer to obtain 1 mL final volume. The pH of PBS buffer was adjusted to pH 8 (PBS8) to balance the acidic acetate buffer pH 5.5 present in fractions. KCl concentration was also increased to 5.4 mM in PBS8. The fractions were finally diluted two fold with PBS8. 50 µL of the diluted solutions (containing carbohydrates) were mixed with 50 µL of clone supernatant. The following steps were performed as described above. Pools (P1-5) were considered for further characterization. Pools were collected, dialyzed and desalted using a column (100 x 1.6 cm) of Sephadex G-10 at 1 mL/min eluted with deionized water and then freeze-dried.

III.2.i Glycan micro-array

mAbs used to probe the array

Supernatants binding to RUP were tested together with nine well-known pectic mAbs -LM5 (Jones *et al.*, 1997); LM6 (Willats *et al.*, 1998); LM13 (Verhertbruggen *et al.*, 2009b); LM16 (Verhertbruggen *et al.*, 2009b); LM18 (Verhertbruggen *et al.*, 2009a); LM19 (Verhertbruggen *et al.*, 2009a); LM20 (Verhertbruggen *et al.*, 2009a); INRA-RU1 (Ralet *et al.*, 2010) ; INRA-RU2 (Ralet *et al.*, 2010)-.

Immobilization of polysaccharides/oligosaccharides

Polysaccharides and oligosaccharides were used to test mAbs binding specificities (Moller *et al.*, 2008; Sørensen *et al.*, 2009; Pedersen *et al.*, 2012). Most of the polysaccharides were dissolved in water. Mannan was dissolved in 4 M NaOH and Gatti gum in 0.5 M NaOH just before printing. Oligosaccharides were coupled to BSA (Roy *et al.*, 1984; Pedersen *et al.*, 2012). Glycans and glyco-conjugates were printed on a nitrocellulose membrane (0.45 µm pore size, Whatman, Maidstone, UK). Microarray robot Microgrid II (Digilab/Genomic Solutions, Huntingdon, UK) equipped with four split spin or solid spins (Digilab) was used for printing. Prior each sample deposition, pins were washed twice. Two different

concentrations (2 mg/mL; 0.4 mg/mL) were spotted in duplicate. Six drops per spot containing 55.2% glycerol, 44% water and 0.8% triton X-100 were deposited onto nitrocellulose membranes. Samples were printed at 19°C (55% humidity). Experiment was repeated twice.

Glycan micro-array

Arrays were laid in a 6 wells ELISA plate for incubation with 2 mL capacity. Arrays were blocked by incubation in 5% skimmed milk PBS buffer (PBS5%) for 2 h at room temperature. Arrays were probed for 2 h at room temperature with characterized mAbs against pectin and 22 hybridoma supernatants diluted 1/10 and 1/5 in PBS5%, respectively. After washing in PBS, arrays were incubated for 2 h at room temperature with anti-mouse or anti-rat secondary mAbs conjugated to alkaline phosphatase (Sigma, Poole, UK) diluted 1/5000 in 5%PBS. After washing, arrays were developed using substrate containing 5-bromo,4-chloro,3-indolyphosphatase (BCIP) and nitroblue tetrazolium (NBT) in BCIP/NBT buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM diethanolamine, pH 9.5).

Scanning and data analysis

Developed arrays were scanned (Cannon 8800, Soborg, Danmark) and converted to negative-image 16-bit grey-scale TIFFs (Moller *et al.*, 2008). TIFF files were uploaded into ImaGene 6.0 microarray analysis software (BioDiscovery, El Segundo, CA, USA). To create an analysis area for each spot, a semi-automatic gridding was used for analysis. Five-pixel zone around each spot was used for calculation of local background signal. Individual spot signals were defined as the mean pixel value within each spot area minus the median pixel value in the surrounding local background area. Signal of the four spots derived from each sample defined a “mean sample value”. The “total mean sample value” (TMSV) is the media of both experiments. For each mAb, the maximal TMSV was set to 100%. All other values were defined according to the maximal TMSV. To avoid exaggerate fold change values, a minimal of 5% of the maximal value was cut off. Data sets from the TMSVs were transferred in online heap mapper software(http://bbc.botany.utoronto.ca/ntools/cgibin/ntools_heatmapper.cgi)

to generate heatmaps. A hierarchical cluster analysis of the ELISA data was performed using TMSV profile data-set in Eclust software (http://ep.ebi.ac.uk/EP/EPCLUST/).

III.2.j LC-MS

Ion pairing-reversed phase chromatography separation (IP-RP-UHPLC)

Chromatographic separation of RUP samples and P1-5 was achieved at 45°C, on a ultra performance liquid chromatographic system (UHPLC, Acquity H-Class® Waters, Manchester, UK), mounted with a BEH C18 column (100 mm × 1mm, packed with 1.7 µm porosity particles; Waters, Manchester, UK), at a flow rate of 150 µL/min. A ternary gradient was applied (Table.III.3).

Table III.3: Ternary gradient applied on LC-MS

Time	%A	%B	%C
0-10 min	73-50	2-25	25
10-23.5 min	50-2	25-73	25
23.5-27.5 min	2	73	25

A: pure water, B: pure methanol and C: 20mM heptylammonium dissolved in water, and pH value adjusted to 6 by addition of formic acid

Collision-Induced Dissociation (CID) MS/MS measurements.

MS/MS experiments were performed on a Q-TOF Synapt G2Si HDMS (Waters, Manchester, UK) in negative ion mode. Samples were diluted 2 fold with MeOH introduced manually into the electrospray ion source at a flow rate of 5 µL/min. Ion spray capillary voltage was maintained at 3 kV and the temperature source at 120°C. Argon was used as collision gas. Collision energy was optimized for each sample. Fragments were annotated according to the nomenclature of Domon and Costello (Domon and Costello, 1988). Data were recorded using MassLynx 4.1 (Waters).

III.2.k Immunofluorescence microscopy

Tissue preparation

Small pieces of fresh tissues were sampled from peripheral regions of potato tubers. Samples were fixed in a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.4) for 20 h at 4 °C. Samples were washed with Na-phosphate buffer before dehydration in a graded series of increasing concentrations of ethanol (30, 50, 70, 85, 95 and 100 ethanol, 1 h for the first five steps and overnight for the last step). Samples were progressively infiltered in LR White (London Resin, Reading, UK) resin according to the following schedule: 20, 40, 60 and 80%, 100% LRW/ethanol, 1 h for each step and then 2 h and overnight in pure resin. The tissue samples were transferred into individual gelatin capsules filled with fresh resin. Resin was polymerized at 55 °C for 2 days. Cross sections (1µm thick) were cut with an ultramicrotome UC7 (Leica Microsystems, Germany) equipped with a diamond knife and mounted on multi-well slides treated with VectaBond adhesive (Vector Laboratories, www.vectorlabs.com).

Arabidopsis thaliana root seedlings growing

Arabidopsis thaliana ecotype 'Columbia ' seeds were sterilized in 70° ethanol for 5 min and washed 5 times with sterile water. Seeds were left 3 days at 4 °C on plates containing Murashige and Skoog salt mixture and 0.215 g/L 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.8 in 0.4 % agar. Subsequently, seeds were allowed to germinate for 7 days. Plates were incubated at 22°C in a growth chamber 16 hours light/8 hours dark cycle. Complete seedlings were harvested for microscopy studies.

Immunolabeling

Immunolabeling of potato tuber cross sections was performed on multi-well glass slides by applying and removing 40 µL droplets of the appropriate reagents. Roots (5 per antibody/mAb) were labelled in 24 wells ELISA plates with a total volume of 500 µL of

reagents. Sections and roots were treated with 3 % BSA in PBS for 1 h at room temperature to block non-specific labeling. Then, material was incubated for 1 h at room temperature with primary mAb (LM5; LM6; INRA-RU1) or hybridoma supernatants at dilutions of 1/10 in PBS containing 1 % BSA and 0.05 % Tween (BT-PBS). Tissues were washed (5 time X 5 min) in BT-PBS and then exposed to goat anti-rat or anti-mouse IgG antibodies conjugated to Alexa fluor® 546 at a dilution of 1/100 in BT-PBS for 1 h. The samples were washed 3 times in BT-PBS and 5 times in water. Roots were examined in a light macroscope equipped with epifluorescence illumination (Nikon intensilight C-HGFIE) and images were captured with a QImaging's EXi Aqua™ camera. Immunofluorescence was observed using a 510-560 nm band-pass excitation filter and emission at > 590 nm. Potato tuber sections were observed using a LEICA DMRD microscope equipped with epifluorescence irradiation. A band-pass filter 515-560 nm was used as excitation filter and fluorescence detected at > 570 nm. Images were recorded with a sensitive cooled camera (Nikon DS-1QM). In some cases, enzymatic treatments were applied on sections prior to immunolabeling. RGI was degraded using arabinanase (50 U/mL), galactanase (50 U/mL) and rhamnogalacturonan hydrolase (0.1mg/mL) in 50 mM acetate buffer pH 4.5, for one night at 37°C. For multi-treatment on a same cross section, each enzyme has been applied one by one, in the present order: first galactanase, then arabinanase then rhamnogalacturonane lyase. After and between each enzymatic treatment, samples were washed first with 50 mM acetate buffer pH 4.5 (5 times X 5 min) and then with water (3 times X 5 min). NaOH treatment was performed for pectin deesterification. Treatment was applied alone or after enzymatic digestion using galactanase. Samples were incubated with 0.05 M NaOH for 30 min at 4 °C and then washed with water (5 times X 5 min). As a control, sections were incubated with the secondary antibody, only.

III.3 Results

III.3.a Oligosaccharides generation and characterization

Raw RGI was solubilized from de-starched potato pulp using alkaline extraction with sodium hydroxide at high temperature (Zykwinska *et al.*, 2006). In these conditions, β-elimination allows HG depolymerization. Raw RGI was recovered from the extract by precipitation with 70% ethanol. 17 g of raw RGI were recovered from 100 g of dry pulp. Gal and GalA were the main sugars and accounted for 42.9 mol % and 41.9 mol % of the total sugars, respectively (Table.III.4). A GalA over Rha molar ratio of 8.2 was calculated, evidencing the presence of residual HG stretches in the sample. Raw RGI was therefore further purified using an anion exchange batch separation. The fraction eluting with 20 mM sodium acetate pH 4.5 was selected based on recovery yield (40%, w/w of raw RGI) and GalA over total neutral sugars ratio (0.18). Purified RGI was particularly rich in Gal and to a lesser extent in Ara and exhibited a GalA over Rha molar ratio of 2.6, in agreement with Øbro *et al*., (2004) findings. Minor amounts of hemicellulose-derived sugars (Fuc, Xyl, Man, Glc) were also detected. The molar ratio of GalA to Rha in purified RGI decreased by a factor of 3 compared to raw RGI, showing that HG fragments were efficiently removed by the anion-exchange purification step.

The purified RGI was then sequentially treated with an endo-galactanase and an endo-arabinanase in order to produce so-called low-branched RGI. The enzymatic treatment allowed the removal of 86.3% (w/w) of the Gal residues and 83.4% (w/w) of the Ara ones (Table.III.4) in agreement with previously reported data (Øbro *et al.*, 2009). The RGI backbone was further split using a rhamnogalacturonan hydrolase and the large fragments were removed by precipitation with 50 % ethanol. The pool of oligosaccharides recovered in the ethanol-soluble fraction (designated RUP oligosaccharides) exhibited a GalA over Rha molar ratio of 1.2 with abundant Gal and to a lesser extent Ara (Table.III.4). RUP oligosaccharides also contain low amounts of Fuc, Xyl, Man, Glc and GlcA, which likely originate from residual hemicelluloses (Cornuault *et al.*, 2014).

Table III.4: Recovery yield (weight %) and sugar composition (mol %) of the different RGI fractions.

	Raw RGI	Purified RGI	Low-branched RGI	RUP oligosaccharides
Yield	100	40.5	13.3	4.9
Rha	5.1	5.1	21.3	22.7
Fuc	nd	1.2	0.4	0.7
Ara	10.0	13.3	6.7	7.9
Xyl	nd	0.9	2.5	1.2
Man	nd	0.8	1.2	0.7
Gal	42.9	64.6	27.0	36.7
Glc	nd	1.2	3.9	0.3
GalA	41.9	13.1	35.5	28.3
GlcA	ND	ND	1.4	1.5

ND: non-determined; nd: not detected.

III.3.b Glycan micro-array

Glycan micro-array (Moller *et al.*, 2008; Pedersen *et al.*, 2012) was used to further characterize the RUP oligosaccharides. Raw RGI, RUP oligosaccharides and a set of selected pectic glycans were printed on an array, which was then probed with a selection of eight mAbs, three being specific to HG domains and five to RGI domains. LM18, LM19 and LM20 bind to HG of various degree of methylation (D

M) (Verhertbruggen *et al.*, 2009a). INRA-RU1 and INRA-RU2 are specific to unbranched RGI backbone (Ralet *et al.*, 2010). LM5 is specific to galactans (Jones *et al.*, 1997) while arabinans are recognized by LM6 and LM13 (Willats *et al.*, 1998; Verhertbruggen *et al.*, 2009b). For each antibody, the level of binding to RUP oligosaccharides was compared to the level of binding to the other glycans (Figure.III.2A). Signal intensity from glycan microarray provides some information about the relative levels of glycan epitope, but cannot be considered as a tool for quantifying absolute levels of a specific structure (Moller *et al.*, 2008). Therefore some antibodies have also been tested in ELISA competitive inhibition to confirm the level of recognition to RUP oligosaccharides.

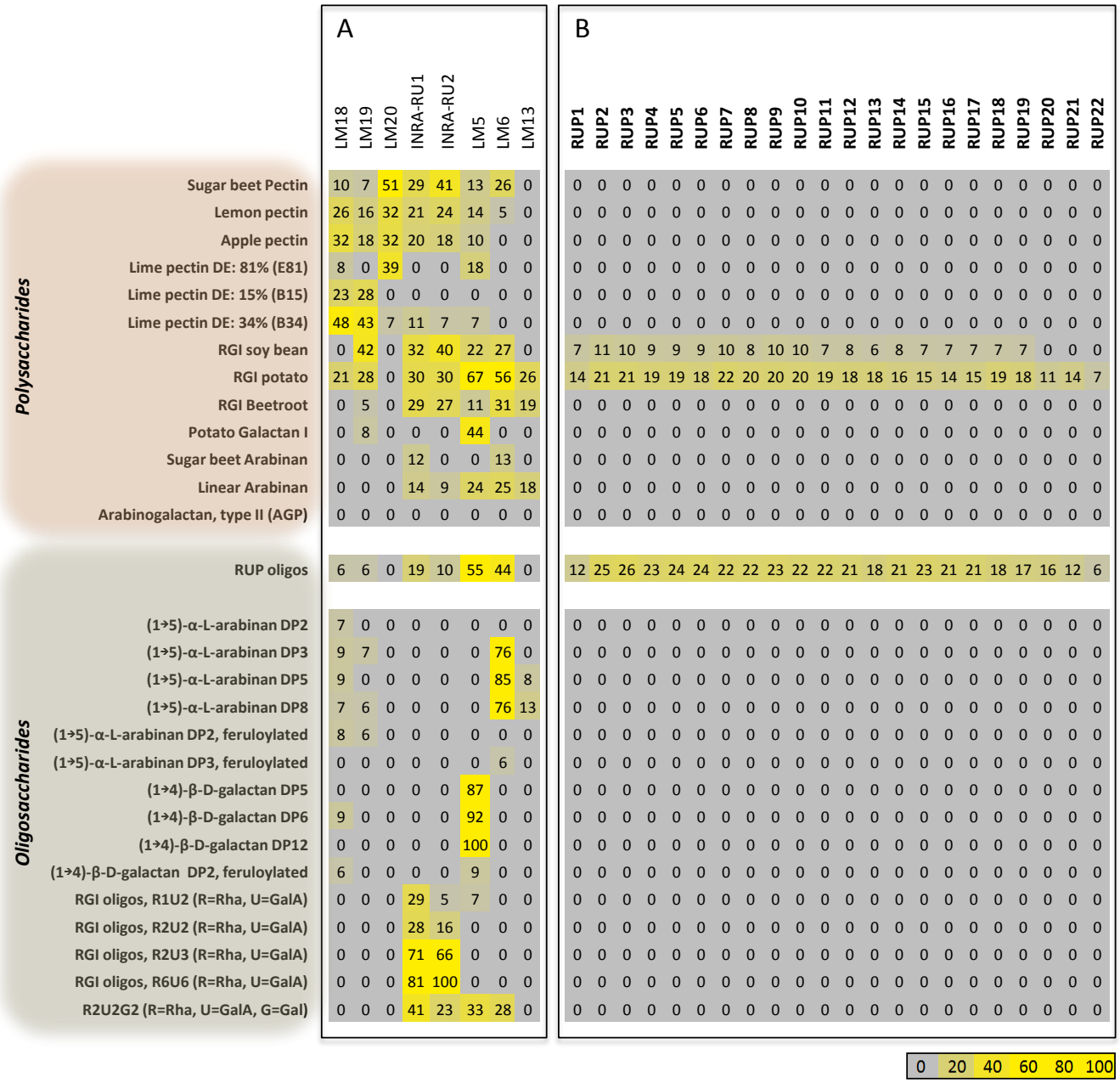


Figure III.2: Heat map glycan micro-array. A: binding of available pectin-related mAbs (LM18, LM19, LM20: HG ; INRA-RU1 and INRA-RU2: RGI ; LM5: (1→4)-galactan ; LM6: (1→5)-arabinan) to RUP oligosaccharides and to a set of purified oligosaccharides and polysaccharides. B: RUP antibodies (RUP1-RUP22) recognition pattern to oligosaccharides and polysaccharides. Spots signals were scored form 0 (grey) to 100 (yellow).

Structure profile of polymeric glycans

mAbs tested on whole pectin samples from sugar beet, lemon, apple and lime revealed a variability in the occurrence of HG and RGI domains in these samples (Figure.III.2A). Indeed, lowly (LM18 and LM19 binding) or highly (LM20 binding) methylated HG domains were systematically revealed whatever the pectic sample. In contrast, RGI backbone (INRA-RU1

and INRA-RU2 binding) was strongly revealed in sugar beet, lemon and apple pectin samples and in only one lime pectin sample with a low intensity signal. LM6 binding was restricted to sugar beet pectin, which is well known to encompass long branched arabinan side chains. LM13 did not bind to any of the pectic samples, precluding the presence of long linear arabinan stretches in these samples. LM5 bound weakly to most of the pectin samples, suggesting the constant presence of galactan side chains.

Three whole RGI domains isolated from soybean, potato and beetroot were also tested (Figure.III.2A). All three RGI samples were recognized, by LM18 and/or LM19. Spot signals for potato and soybean RGI were particularly intense. These results are consistent with the high GalA content of these fractions and strongly suggest the presence of large amounts of residual HG domains (Table.III.1). All three RGI samples were also strongly recognized by INRA-RU1 and/or INRA-RU2, which is consistent with the presence of unsubstituted or lowly substituted RGI backbone stretches. Finally, side chains-related mAbs (LM5, LM6 and LM13) bound to virtually all the RGI samples tested. The only exception is the absence of LM13 binding to soybean RGI.

Finally, four isolated pectic side chains (galactan, branched arabinan, linear arabinan and type II arabinogalactan) were tested (Figure.III.2A). Beside expected pectic side chains-related mAbs binding (LM5 binding to potato galactan I, LM6 binding to sugarbeet branched arabinan and both LM6 and LM13 binding to linear arabinan), some unexpected binding occurred revealing the presence of (i) residual HG domains in galactan and branched arabinan, (ii) residual RGI backbone in the two arabinan samples, (iii) long stretches of linear arabinan in branched arabinan, and (iv) galactan stretches in linear arabinan. As anticipated, type II arabinogalactan was not recognized by any of the mAbs tested.

Structure profile of oligomeric glycans

RUP oligosaccharides were recognized by most of the available mAbs, except LM20 and LM13, precluding the presence of highly methylated HG stretches and long linear arabinan stretches (Figure.III.2A). LM18 and LM19 had a very weak affinity to RUP oligosaccharides, which is consistent with the very low occurrence of residual HG stretches in this fraction, as reveled by sugar analysis. (Table.III.4). INRA-RU1 and INRA-RU2 did bind to RUP

oligosaccharides but the signal intensity was lower than that observed for raw RGI. The unbranched backbone from the RGI polymer has indeed been at least partly digested by rhamnogalacturonan hydrolase. ELISA competitive inhibition assays performed with RU and RUP oligosaccharides confirmed the low affinity of INRA-RU1 for RUP oligosaccharides (Figure.III.2A). The moderate affinity of INRA-RU1 for RUP oligosaccharides strongly suggests that the RU backbone is highly branched. Unexpectedly, glycan microarray assays showed that both LM5 and LM6 strongly bound to RUP oligosaccharides (Figure.III.2A).

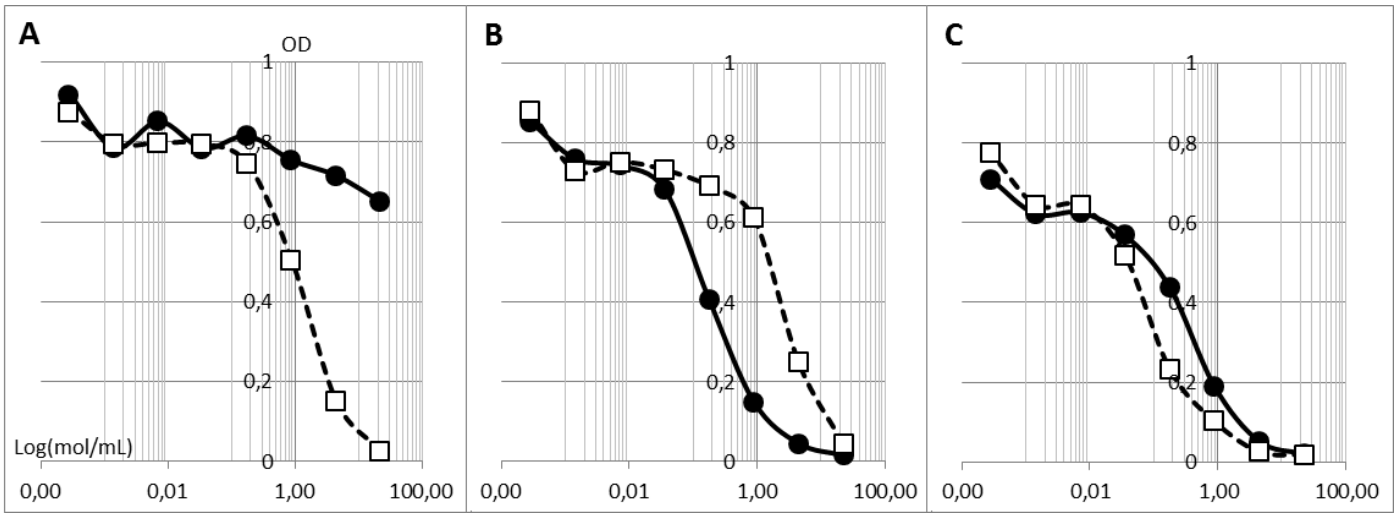


Figure III.3: ELISA competitive inhibition. Optical density (OD) decreases according to the concentration of the antigen used. Recognition comparison of RUP oligosaccharides (⊣●⊢) to each respective mAb epitope (A: INRA-RU2/RU-oligosaccharides; B: LM5/galactan-oligosaccharides; C: LM6/arabinan-oligosaccharides (⁻⁻□⁻⁻)). RUP oligosaccharides are lowly recognized by INRA-RU2. In contrast, LM5 and LM6 have a high affinity to the RUP oligosaccharides.

ELISA competitive inhibition assays (Figure.III.3B and III.3C) confirmed the strong binding of LM5 and LM6 to RUP oligosaccharides. Furthermore, LM5 and LM6 showed a similar affinity to RUP oligosaccharides than to their respective epitopes (galactan and arabinan). In order to get further insight into the significance of INRA-RU1, INRA-RU2, LM5 and LM6 binding to RUP oligosaccharides, 15 purified RGI-related oligosaccharides were printed on an array according to Pedersen *et al*., 2012 (Figure.III.2A). As previously reported (Ralet *et al.*, 2010), INRA-RU1 and INRA-RU2 bound strongly to unbranched RU oligosaccharides, the avidity increasing with DP increase. A rather strong binding to R2U2G2 (α-L-Rha*p*-(1→4)- α-D-Gal*p*A-(1→2)- α-L-Rha*p*-(1→4)-D-Gal*p*A substituted by one Gal residue on each Rha residue) was also observed, in agreement with previously published data (Ralet *et al*., 2010). As expected, LM5 bound strongly to galactan oligosaccharides and LM6 to arabinan oligosaccharides of DP≥3

(Jones *et al.*, 1997; Willats *et al.*, 1998). More unexpected was the rather strong binding of both mAbs to R2U2G2.

Considering the endo-galactanase and endo-arabinanase treatment applied to purified RGI and the removal of large fragments after rhamnogalacturonan hydrolase action, it is unlikely that RUP oligosaccharides still encompass long arabinan and galactan side chains or long unbranched RU stretches. It is more likely that RUP oligosaccharides mainly consist in rather short RU fragments highly branched with short Ara- and/or Gal-containing side chains that appear to be recognized by INRA-RU1, INRA-RU2, LM5 and LM6.

III.3.c Production of monoclonal antibodies to RUP oligosaccharides

Immunization of mice with RUP-OVA was effective in inducing a strong immune response. After lymphocyte fusion with splenocytes, hybridomas were screened on RUP-BSA and BSA. The 22 clones (RUP1-RUP22 antibodies; Figure.III.2B), which reacted only with RUP-BSA, were selected for further characterization. Isotyping revealed that the selected mAbs were all IgMs.

III.3.d Epitope characterization

The binding of the 22 supernatants to a set of cell-wall derived polysaccharides and oligosaccharides was investigated using glycan microarray (Figure.III.2B). All selected supernatants did not bind to non-pectic oligosaccharides and polysaccharides printed on arrays (data not shown). RUP1-RUP22 exhibited similar epitope recognition patterns that differed widely from those obtained with well-known pectin antibodies. The two sole polysaccharides recognized by the supernatants were potato and soybean RGI. This specific binding confirms that the epitope is part of the RGI domain. RUP1-RUP22 antibodies all bound to the RUP oligosaccharides used for mice immunization and clone selection but all other RGI-related oligosaccharides were not recognized. Therefore, it may be hypothesized that the epitope corresponds to a very specific structure inside side chains or between RGI side chains and backbone.

To better understand the structure recognized by RUP antibodies, complementary ELISA competitive inhibition assays were performed. The lower the inhibition concentration 50 % (IC50%) is, the higher the affinity of the antibody for the inhibitor is. HG, type II arabinogalactan and R2U2G2 were tested as 3 negative controls (Table.III.5). Since all mAbs exhibited similar binding profiles on microarray, one mAb was selected: RUP2 antibody. This antibody showed a stronger affinity for potato raw RGI (IC50: 0.05 ug) than for RUP oligosaccharides (IC50: 0.15 ug). The removal of HG domains and enzymatic hydrolysis of linear arabinan, galactan and unbranched RU backbone probably led to partial removal of the epitope.

Table III.5: ELISA competitive inhibition. Préciser anticorps et antigènes

Glycans	IC50% (µg/ml)
HG-citrus	NI
R2U2G2	NI
AGII	NI
Potato galactan II	NI
Lupin galactan	20
Potato pectic galactan	1
RUP oligosaccharides	0.15
Raw RGI-potato	0.05
RGI-carrot	0.05

IC50% is defined as the concentration of inhibitor required to give 50% inhibition of the binding of the antibody to RUP-BSA. The lower the IC50% is, the higher the inhibition is. NI: No Inhibition.

Carrot RGI and, to a lower extent, lupin galactan and commercial potato pectic galactan were recognized by the RUP2 antibody. All these substrates originate from galactan-rich organs: tubers (for potato and carrot) and seed cotyledons (for lupin) (Jarvis *et al.*, 1981; Massiot *et al.*, 1988; Buckeridge *et al.*, 2005). Interestingly, the RUP2 antibody did not have the same avidity for the two galactan samples extracted from potato. The epitope occurred only in the commercial galactan, which is less pure. Indeed, the commercial potato pectic galactan (IC50 1ug) has larger amounts of contaminating sugars (Rha; Ara; GalA) than potato galactan II (Table.III.1). Altogether, the ELISA competitive inhibition results suggest that the

epitope is present in galactan-rich sources and that sugars other than Gal are involved in the recognition.

III.3.e RUP fractionation by anion-exchange chromatography and fraction analysis

An epitope detection chromatography (EDC) assay, adapted from Cornuault *et al*. (2014) was set-up. RUP oligosaccharides were fractionated according to their charge density by anion-exchange chromatography, (Figure.III.4). Each fraction was analyzed for its GalA and total neutral sugars content and was tested with RUP2 antibody using an ELISA competitive inhibition assay. Each chromatographic fraction was used as a competitor in antibody-antigen mixtures. Standards were prepared by mixing serial dilution of RUP oligosaccharides with the antibody. RUP-BSA was used to coat 96 wells plates. Uncoated sites were saturated and antibody-antigen mixtures were added. After washing, secondary antibodies were added and plates were developed. The competitor efficiency was evaluated by absorbance reading, the more efficient the competitor, the lower the absorbance.

Five pools (designed P1-P5) were identified based on GalA and neutral sugars analyses and EDC results. P1, P2 and P5 represent a very low proportion of total RUP oligosaccharides. Nonetheless, P1, containing neutral sugars only, P5, containing high proportions of GalA and, to a lesser extent, P2, containing both acid and neutral sugars, were particularly well recognized by the antibody. P3 and P4, containing both acid and neutral sugars, represent a high proportion of total RUP oligosaccharides. Since the decrease in OD has to be related to the sugar concentration of each pool, P3 can be considered as poorly recognized and P4 as moderately recognized by the new mAb.

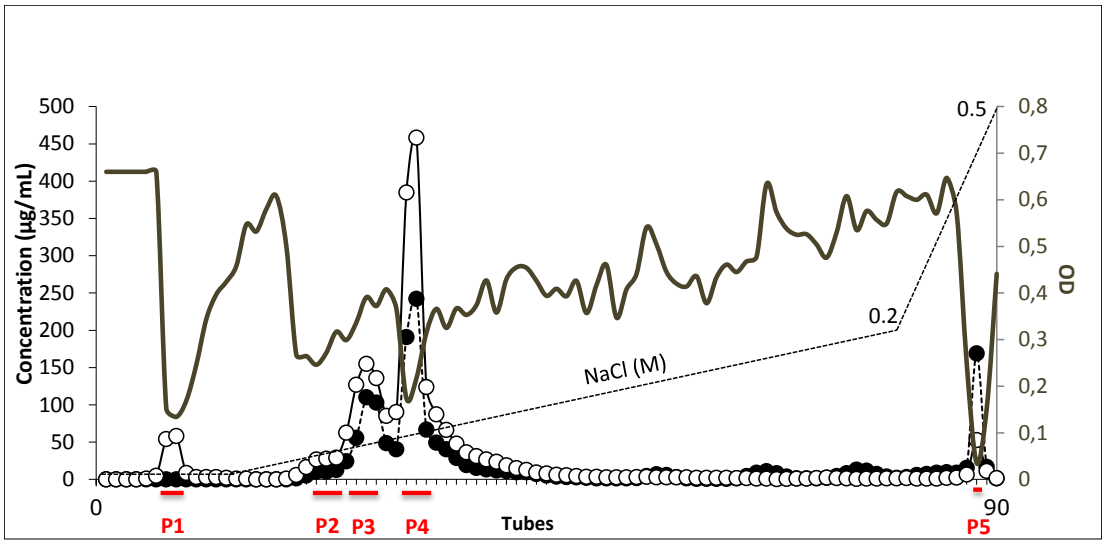


Figure III.4: Fractionation of RUP oligosaccharides using anion exchange chromatography and ELISA competitive inhibition on the 90 using fractions as competitorsseparated by DEAE chromatography. (---●---) Uronic acid concentration ; (—○—) neutral sugars concentration; (-------) NaCl gradiant for the DEAE ; (▬) optical density (OD). Five different pools were definded P1-P5.

Linkage analysis

Linkage analysis was performed on RUP oligosaccharides and on pools P1-P4 (Table.III.6). P5 could not be analyzed because it contained too low levels of neutral sugars. Sugar linkage analysis of RUP oligosaccharides was consistent with the neutral sugar composition determined as alditol acetates (Table.III.4). Close to 50% of the Rha*p* residues were identified as 2,4-linked units and 16.8% as 2-linked ones, showing that a high proportion of internal Rha residues linked at *O*-2 to GalA residues within the RGI stretches are branched at *O*-4 with side chains. A high proportion (27.8%) of 4-linked Rha*p* was detected. These units are assumed to constitute the non-reducing ends of RGI fragments branched at *O*-4 with neutral sugars side chains. Finally, a low proportion of unbranched terminal Rha*p* was detected. Considering 2- and 2,4-linked Rha*p* as internal residues and t- and 4-linked Rha*p* as non-reducing termini, an average DP of 6 can be calculated for the RGI backbone in RUP oligosaccharides. Arabinose was mainly present as 1,5-linked Ara*f* and t-Ara*f* units, which is typical of linear arabinans (Khodaei and Karboune, 2013). Terminal Ara*p* was also detected as previously mentioned for soybean RGI (Huisman *et al.*, 2001). The ratio of 1,5-linked Ara*f* over t-Ara*f* + t-Ara*p* is close to 1. Galactose was mainly present as t-Gal*p*. The low ratio of 1,4-linked Gal*p*/Gal*p*T (0.3) indicates that Rha*p* units mainly carry single Gal*p* units. RUP oligosaccharides also contain 1,6-Gal*p* and 1,3-Gal*p* but these linkages were detected in very

low amounts in agreement with Khodaei and Karboune (2013). Considering the proportion of branched Rha*p* residues (75%) and the proportion of total Ara and Gal residues, one can assume that RUP oligosaccharides encompass highly branched RU oligosaccharides (average DP6) bearing monomeric or very short Ara and Gal-containing side-chains.

Table III.6: Linkage analysis of RUP oligosaccharides and related anion-exchange chromatography-resolved pools P1-P4.

Permethylation	Linkages	RUP		P1		P2		P3		P4	
		%/∑ ether	% family	%/∑ ether	% family	%/∑ ether	% family	%/∑ ether	% family	%/∑ ether	% family
234 Rhamnose	t-Rha	2,4	6,6	0	0	0	0	0,4	1,1	2,4	7,3
34 Rhamnose	2Rha	6,3	16,8	0	0	0	0	0	0	5,3	15,8
23 Rhamnose	4Rha	10,3	27,8	0	0	9,1	40,1	15,6	46,5	7,8	23,3
3 Rhamnose	24Rha	18,1	48,8	0	0	11,8	52,3	17,2	51,2	17,4	51,8
Rhamnose	234Rha	0	0	0	0	1,7	7,6	0,4	1,2	0,6	1,7
Total		37,1	100	0	0	22,6	100	33,7	100	33,6	100
235 Arabinose	t-Ara*p*	4,2	30,7	0	0	1,8	6,6	2,8	9,7	6	24,7
234 Arabinose	t-Ara*f*	1,8	13	0	0	8	29,4	11,5	40,1	5,6	22,7
23 Arabinose	5Ara	7,8	56,3	40,1	100	17,4	64	13,9	48,1	12,1	49,7
2 Arabinose	35Ara	0	0	0	0	0	0	0,6	2,1	0,7	2,9
Total		13,9	100	40,1	100	27,2	100	28,8	100	24,4	100
234 Xylose	t-Xyl	2,4	100	0	0	2	100	0	0	0,8	100
Total		2,4	100	0	0	2	100	0	0	0,8	100
2346 Galactose	t-Gal	29,3	64,9	21,4	35	22,8	47,3	33,1	88	29	70,9
246 Galactose	3Gal	3	6,6	0	0	1,7	3,6	1,8	4,8	1,6	3,9
234 Galactose	6Gal	2,3	5,1	0	0	2,7	5,5	0,3	0,7	1,6	4,1
236 Galactose	4Gal	9,4	20,9	38,5	65	17,1	35,4	1,9	5,3	8,1	20
26 Galactose	34Gal	0,5	1,2	0	0	2,4	4,9	0,2	0,5	0,1	0,4
23 Galactose	46Gal	0,6	1,3	0	0	1,6	3,3	0,3	0,8	0,3	0,7
Total		45,1	100	59,9	100	48,2	100	37,6	100	40,8	100
2346 Glucose	t-Glc	1,4	100	0	0	0	0	0	0	0,4	50
Total		1,4	100	0	0	0	0	0	0	0,4	50

P1 appeared solely composed of Ara*f* and Gal*p* (Table.III.6). This pool that is very well recognized by the RUP2 antibody (Figure.III.4), thereby encompasses free pectic side chains that are not attached to a RU backbone. Surprisingly, solely 5-linked Ara*f* units and no t-Ara*f* or t-Ara*p* units were detected. Galactose was present as 4-linked and terminal units. Since neither branching points nor terminal Ara units were detected, 5-linked arabinan stretches shall be included into a 4-linked galactan chain. Considering the proportion of 5-linked Ara*f*, 4-linked Gal*p* and t-Gal*p*, the following overall structure can be proposed:

$$[(1\rightarrow4)\text{-}\beta\text{-D-Gal}p]_x\text{-}[(1\rightarrow5)\text{-}\alpha\text{-L-Ara}f]_y\text{-}[(1\rightarrow4)\text{-}\beta\text{-D-Gal}p]_z$$

RUP oligosaccharides were recovered after enzymatic treatment of the purified potato RGI polymer by endo-galactanase and endo-arabinanase, and since t-Gal is present in large amounts, it is unlikely that x, y and z exceed a value of 3.

P2, P3 and P4 were roughly similar with respect to Rha/Ara/Gal proportion. In P2 and P3, a vast majority (99-100 mol%) of the Rha*p* units were branched at *O*-4 and close to 50% of the total Rha can be attributed to terminal non-reducing ends (terminal and 4-linked Rha*p*). Thus, it can be assumed that the oligosaccharides present in P2 and P3 have RU backbone of DP4. In contrast, close to 25% of the Rha*p* units present in pool 4 are unbranched (terminal and 2-linked) and an average DP of 6 could be calculated for the RU backbone in this pool. The average length of side chains differed significantly between P2, P3 and P4. P2 contains a much higher proportion of internal Gal units (mainly 4-linked Gal*p* but also 3-, 6-, 3,4- and 4,6-linked Gal*p*) than P3 and P4, which contain mainly t-Gal*p*. P2 also contains a slightly higher proportion of internal 5-linked Ara*f* units compared to P3 and P4. Altogether, Ara- and Gal-containing side chains are shorter in P3 (average DP 1.5) and P4 (average DP 1.6) compared to P2 (average DP 2.5).

Mass spectrometry analysis of RUP oligosaccharides

Linkage analysis showed that the RGI backbone length in P2-4 was low (DP 4-6). Substitution by short Ara*f*-*p* and Gal*p*-containing side chains was also evidenced. Mass analysis of RUP oligosaccharides was performed by LC-MS. Nine different peaks (A-I) were observed (Figure.III.5). Spectra from peaks A-I were analyzed and 11 RGI-related oligosaccharides were detected in peaks A-H (Supplementary Figure.III.1-2). Oligosaccharides encompassing a RU backbone of DP4 and 6 were observed. Oligosaccharides with a DP4 backbone were eluted first in peaks A-C. Then elution of oligosaccharides with a DP6 backbone was observed in peaks D-H. Two compounds could be attributed to unbranched RU oligosaccharides, 6 compounds to RU oligosaccharides branched solely with hexose units and 3 compounds to RU oligosaccharides branched with both hexose and pentose units. Based on linkage analysis (Table.III.6), pentose residues are likely to be Ara and hexose residues Gal. In the two major peaks A and E, compounds with molar masses compatible with the presence of a RU backbone (DP4 and DP6) fully branched (presumably on Rha units) with Gal units were

detected. It is noteworthy that R2U2G2 has been detected as a major compound in the rhamnogalacturonan hydrolase digest of potato deesterified modified hairy regions (Schols *et al.*, 1994).

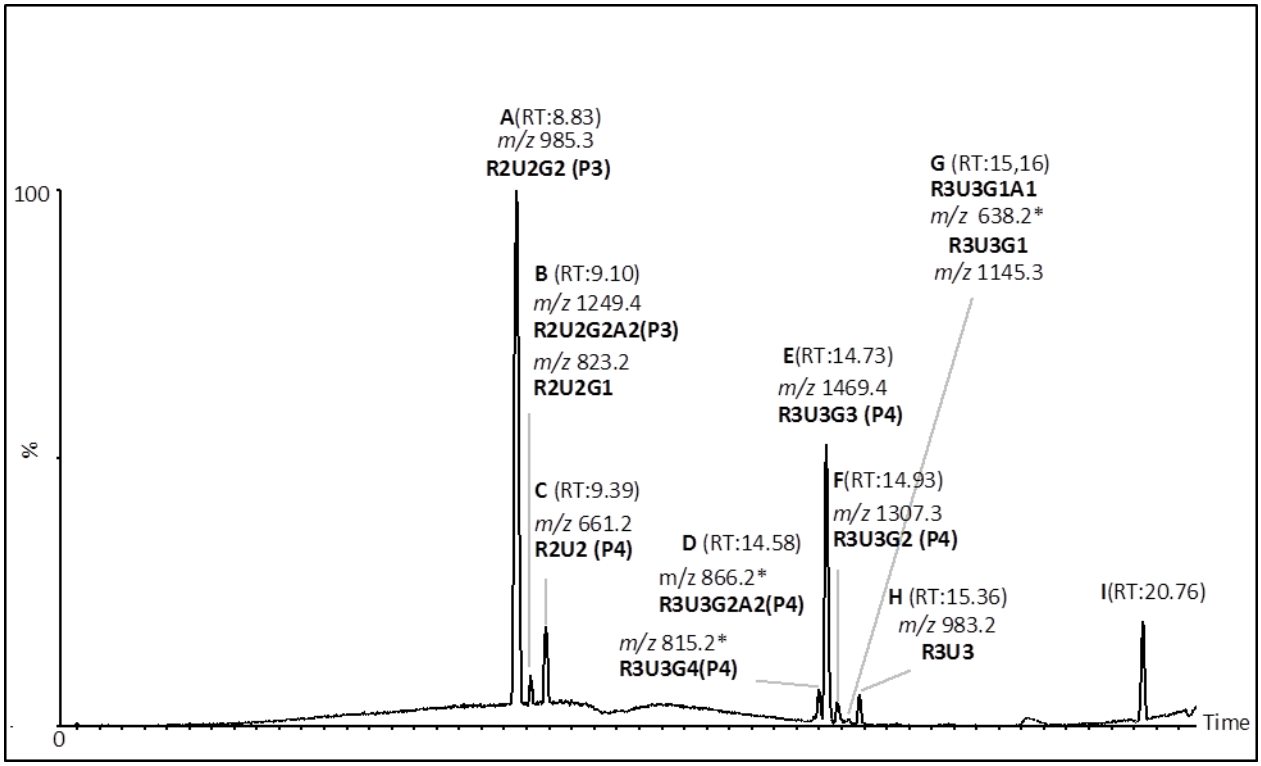


Figure III.5: Separation of RUP oligosaccharides on LC-MS. Nine compounds are detected in the RUP oligosaccharides. Two were found present in pool 3 (P3) and four in pool 4 (P4). R: Rha ; U: Galacturonic acid; G: Gal; A: Ara; * doubly charged ions. RT: retention time.

The two main pools (P3 and P4) recovered by anion-exchange chromatography were run through the LC-MS (Supplementary Figure.III.3). Pools P1, P2 and P5 were unfortunately not recovered in sufficient amounts to allow efficient LC-MS analysis. Seven out of the eleven compounds identified in unfractionated RUP oligosaccharides were detected in pools P3 and P4. From linkage analysis, it could be hypothesized that P3 and P4 were mainly composed of a DP4 and DP6 RU backbone, respectively. For both pools, the RU backbone appeared highly branched at *O*-4 of Rha units, mainly by single Gal*p* residues. In agreement with linkage analysis results, the major compounds identified in P3 and P4 were R2U2G2 (*m/z* 985) and R3U3G3 (*m/z* 734, doubly charged), respectively. In P3, a minor compound at *m/z* 1249 (R2U2G2A2) was also identified. In P4, four minor compounds were identified at *m/z* 661

(R2U2), *m/z* 866 (R3U3G2A2, doubly charged), *m/z* 815 (R2U2G4, doubly charged) and *m/z* 653 (R3U3G2, doubly charged).

The two oligosaccharides present in P3 (R2U2G2, *m/z* 985 and R2U2G2A2, *m/z* 1249) were structurally characterized by MS/MS (Figure.III.6-7). Glycan microarray and ELISA competitive inhibition assays showed that R2U2G2, which is the major compound in P3, was not recognized by the RUP2 antibody. Considering EDC results (Figure.III.4), R2U2G2A2 is presumably poorly recognized either but resolving its structure could give insight into the putative minimal structure recognized (Figure.III.4, Table.III.6).

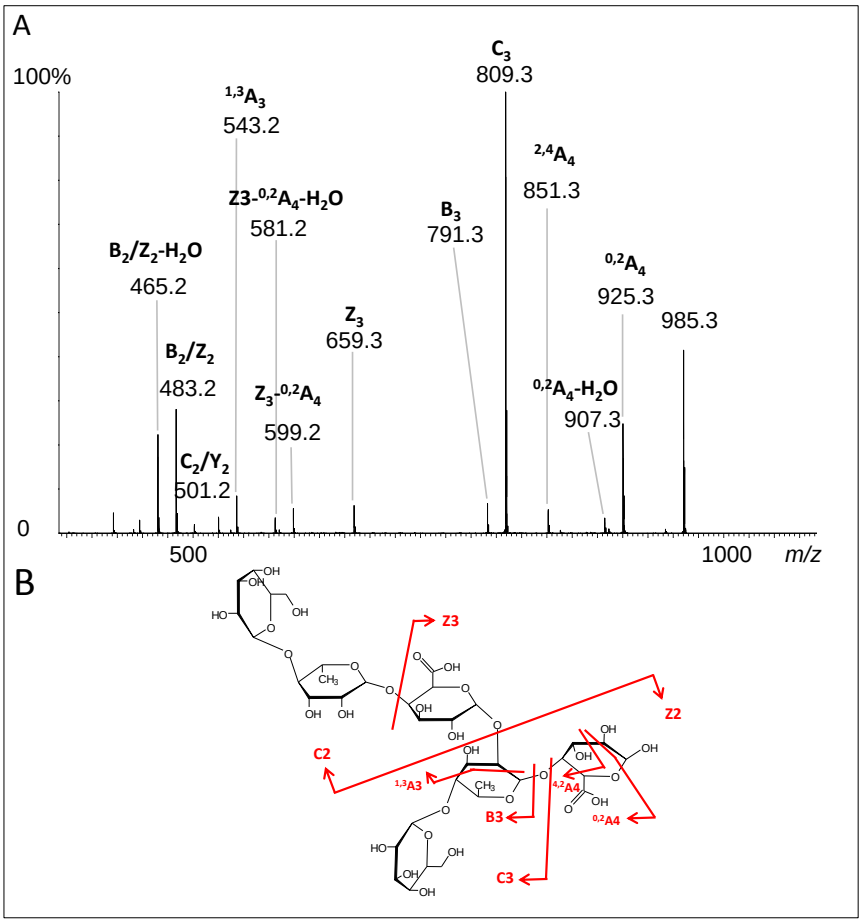


Figure III.6: MS/MS spectrum from the ion at *m/z* 985.3 detected in pool 3 (A) and its respective fragmentation scheme (B).

The isolated ion at *m/z* 985.3 (R2U2G2) generated several fragments in tandem MS/MS (Figure.III.6A). The presence of the ($^{2,4}A_4$) ion at *m/z* 851.3 provides evidence that the reducing end of the molecule consists in a GalA unit. Masses at *m/z* 501.2 (C_2/Y_2) and 483.2

(B_2/Z_2), which have only a water loss difference (*m/z* 18), correspond to the two ions generated when the parent oligosaccharide is fragmented into two DP3 pieces. These ions can be both assigned to fragments containing one Rha, one GalA and one Gal, which unambiguously indicates that the parent ion is a symmetric molecule. Therefore, the two Gal residues are not linked one to another but branched each on a Rha unit. The attribution can be confirmed with the *m/z* 659.3 (Z_3) fragment corresponding to the parent ion having lost a Rha residue on which a single Gal unit is branched. Furthermore, from linkage analyses, it can be deduced that Gal units are mainly linked on Rha residues at *O*-4 (Table.III.6 - as represented on Figure.III.6B-).

Fragmentation of the parent ion at *m/z* 1249.4 (R2U2G2A2) by tandem MS/MS showed that this molecule is not symmetrical (Figure.III.7A-B). Indeed the ion C_2 at *m/z* 501.2 corresponds to a fragment containing one Rha, one GalA and one Gal unit, whereas the Z_2 ion at *m/z* 747.3, contains in addition to the same three sugars, the two Ara residues. The detection of a $^{2,4}A_4$ ion at *m/z* 1115.3 provides evidence that the reducing end of the molecule consists in GalA. The Z_3 ion at *m/z* 923.3 has lost a fragment containing one Rha and one Gal at the non-reducing end of the molecule. These results show that the two Ara residues and one Gal residue are branched onto the internal Rha residue next to the reducing GalA unit. The Gal and Ara residues could be included in a single side chain or in two distinct ones branched at *O*-3 and *O*-4 of the Rha residue. The presence of the ion generated from an intracyclic fragmentation at *m/z* 543.2 ($^{1,3}A_3$) provides evidence that the internal Rha*p* unit is not branched at *O*-3. Indeed, this ion corresponds to a trimer of Gal, Rha and GalA carrying a fragment of the internal Rha*p* residue containing C-2 and C-3 (Figure.III.7B). Therefore, the internal Rha unit is solely branched at *O*-4 with a trimeric chain encompassing one Gal and two Ara residues. The two Ara residues are likely to be branched as a dimer on the Gal unit since the two pentose units are systematically released together after fragmentation. The ion assigned to Z_3 having lost the two Ara units was detected at *m/z* 659.3. Similarly, the two ions at *m/z* 599.3 (Z_3-$^{1,3}A_3$-2Ara) and *m/z* 581.2 (Z_3-$^{1,3}A_3$-H_2O_2-2Ara) do not contain the pentose dimer. However, from the fragmentation pattern (Figure.III.7A-B), it is not possible to determine the nature of the link between the Gal residue and Ara dimer.

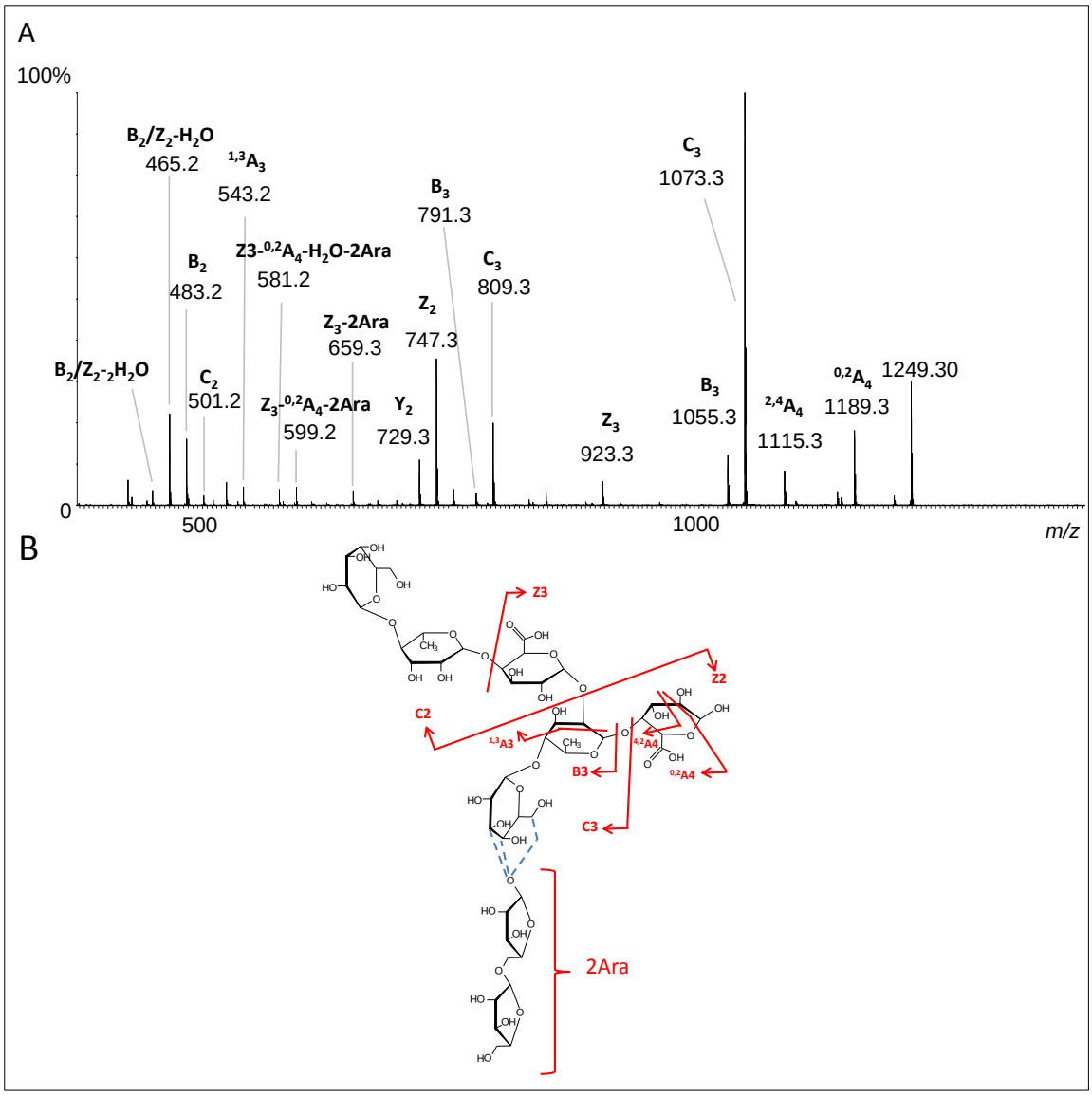


Figure III.7: MS/MS spectrum from the ion at *m/z* 1249.3 detected in pool 3 (A) and its respective fragmentation scheme (B).

III.3.f Labeling on plant tissues

The capacity of the RUP2 antibody to bind pectin *in situ* was studied using transverse sections of potato tuber, with a focus on the periderm region, and Arabidopsis seedling roots. In transverse sections of potato tuber, the labeling pattern of the RUP2 antibody was compared with that of available RGI-related antibodies (LM5, LM6 and INRA-RU1). Potato tuber is galactan-rich (Jarvis *et al.*, 1981) and transverse sections were strongly labeled with LM5 (Figure.III.8A). Epiderm was the only tissue, which was not labeled by LM5. There was a

gradient of the epitope abundance across the phellem and labeling was abundant in the phelloderm and inner parenchyma (Figure.III.8B-8C).

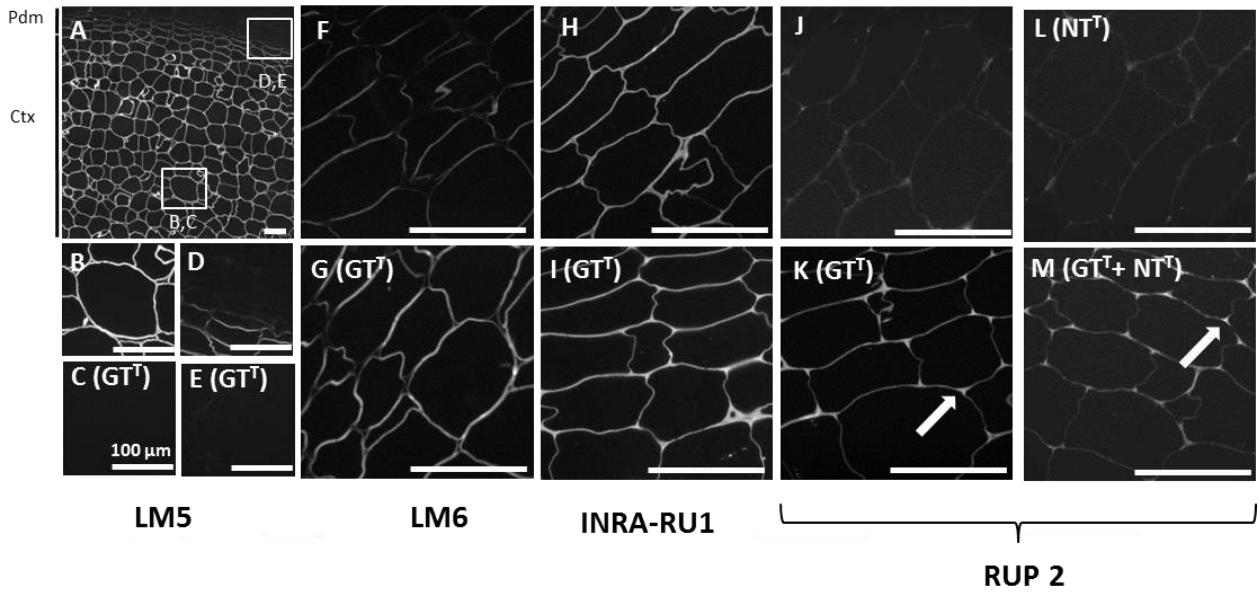


Figure III.8: Labeling of periderm and cortical tissues of potato tuber by available mAbs against RGI. B,C: cortical tissues (Ctx); D-M: pericarp tissue (Pdm); A-E: labeling with LM5; F-G : labeling with LM6; H-I; labeling with INRA-RU1; J-M: labeling with RUP2; L-M: NaOH treatment (NT^T); D,E,G,I,K,M: Galactanase treatment (GT^T). resumer l'information

These results are in agreement with those reported by Bush and McCann (1999) and Sabba and Lulai (2005). The walls from periderm labeled moderately with LM6 (Figure.III.8F) as previously described in previous studies (Bush and McCann, 1999; Sabba and Lulai, 2004). INRA-RU1 bound the periderm walls (Figure.III.8H) while no labeling was observed with RUP2 (Figure.III.8J). As long galactan side chains may impede the access of antibodies other than LM5 to their epitopes, sections were treated with a galactanase prior to immunolabeling procedure. The decrease of labeling with LM5 after galactanase pre-treatment confirmed the efficiency of galactan digestion (Figure.III.8E). The labeling with the three antibodies, LM6, INRA-RU1 and RUP2 was increased (Figure.III.8G; 8I; 8K). With LM6, cell walls were labeled equally (Figure.III.8G). With INRA-RU1 (Figure.III.8I) and RUP2 (Figure.III.8K), tri cellular junctions were more densely labeled in comparison with the intervening walls. The labeling with RUP2 seemed to be present at the intracellular spaces, the middle lamella and primary cell wall, but this has to be confirmed by electronic microscopy. RGI backbone in potato tuber is highly acetylated (Schols and Voragen, 1994; Ishii, 1997) and it has been previously shown that the presence of acetyl groups may restrict

the binding of antibodies to their epitopes (Marcus et al., 2010). In order to remove acetyl groups, NaOH pretreatment was applied to sections untreated or pre-treated with galactanase. In absence of galactanase pre-treatment, no labeling was observed with RUP2 (Figure.III.8L). In galactanase pre-treated sections, NaOH pre-treatment did not increase labeling (Figure.III.8M). Altogether, these results suggest that, as previously inferred from EDC and linkage analyses results, the RGI backbone is not involved in the epitope recognition.

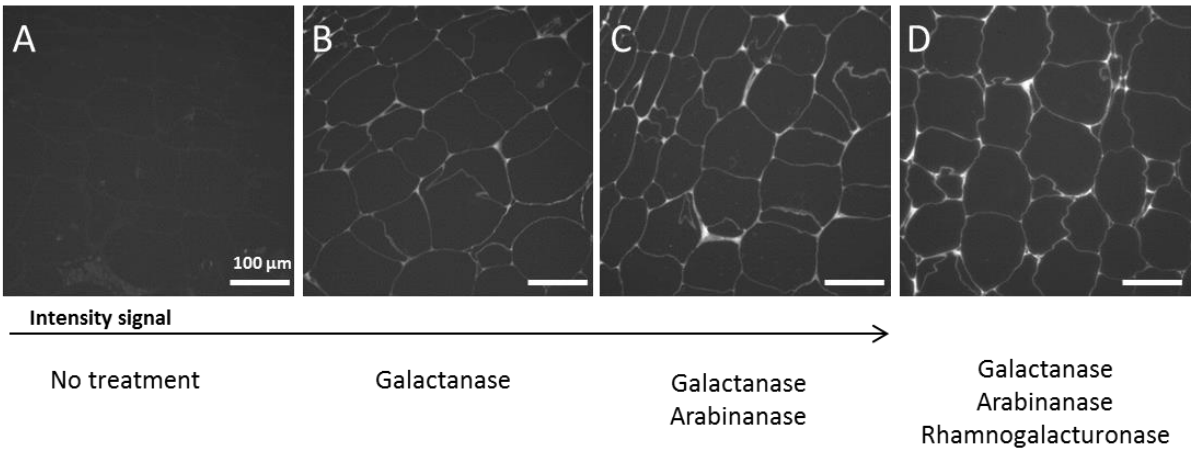


Figure III.9: RUP2 labeling in transverse section of potato tuber. A: no treatment; B: galactanase treatment; C galactanase + arabinanase treatment; D: galactanase + arabinanase + rhamnogalacturonan hydrolase treatment. resumer l'information

To further investigate the impact of RGI traits in the RUP2 binding *in situ*, sections pre-treated with galactanase were pre-treated with an arabinanase alone or in combination with a rhamnogalacturonan hydrolase prior to immunolabeling. The binding of RUP2 was slightly increased with no change in the labeling pattern (Figure.III.9A-D). It could be concluded that the epitope is not sensitive to the enzyme actions and that the degradation of arabinan side chains or the splitting of the RGI backbone slightly increased the antibody access to the epitope. *Arabidospis thaliana* seedling roots have been used as model system for the study of cell development (Koornneef and Meinke). They offer the advantage to be directly amenable to immersion immunofluorescence. We applied RUP2 at the surface of intact *Arabidopsis thaliana* seedling roots grown at 7 days (Figure.III.10). Previously, the LM5

epitope has been shown to occur abundantly in the elongation zone of Arabidopsis roots while LM6 was found abundant at the root apex (Verhertbruggen *et al.*, 2009b). The RUP2 epitope was detected in the elongation zone. In contrast to what was observed with potato tubers, no pre-treatment was required to reveal it.

Figure III.10: RUP2 antibody labels the meristematic and the beginning of the elongation zone in *Arabidopsis thaliana* seedling roots.

III.4 Discussion

III.4.a Production and characterization of RUP oligosaccharides

RUP oligosaccharides were produced from potato pulp. An alkali-soluble raw RGI fraction was recovered. Raw RGI was depleted from residual HG stretches by anion-exchange chromatography and further treated by endo-galactanase and endo-arabinanase to produce a low-branched RGI fraction. Finally, the low-branched RGI fraction was hydrolyzed by a rhamnogalacturonan hydrolase, generating a pool of low-branched RGI oligosaccharides. Large oligosaccharides were removed by precipitation with ethanol. The ethanol-soluble fraction (designated RUP oligosaccharides) represented approximately 5 % of the raw potato RGI (Table.III.4). Linkage analysis showed that RUP oligosaccharides had a DP 4 to 6 RU backbone and that about 75 % of the Rha*p* units were branched by DP1-3 Ara- and Gal-containg side chains, on average. The binding of available mAbs recognizing pectin motifs to

RUP oligosaccharides was evaluated using glycan microarray. INRA-RU1 and INRA-RU2, specific to unbranched RGI backbone, moderately bound to RUP oligosaccharides, which could be explained by the presence of some unbranched RGI stretches in the sample. Surprisingly, LM5 and LM6, binding to β-(1,4)-linked-galactan and α-(1,5)-arabinan of DP ≥ 3, respectively, had a high affinity for RUP oligosaccharides. In order to get further insight into the significance of INRA-RU1, INRA-RU2, LM5 and LM6 binding to RUP oligosaccharides, 15 purified RGI-related oligosaccharides were printed on an array. All four antibodies showed strong binding to R2U2G2, a DP4 RU backbone substituted by one Gal residue on each Rha residue. This oligosaccharide and structurally-related ones are very likely to be largely present in RUP oligosaccharides.

To have another view of the structure of RUP oligosaccharides, analysis by LC/MS was performed. Eleven different RGI-related oligosaccharides were detected. The structure of all fragments contained, as predicted, a DP4 or a DP6 RGI backbone. Some of the fragments were found unbranched (R2U2; R3U3). These motifs have been identified in literature after rhamnogalacturonan hydrolase digestion of RGI substrates of various plant origin (Schols *et al.*, 1994; Zheng and Mort, 2008). Branched RGI oligomers were also detected. Most of the branched oligosaccharides contained Gal residues only (R2U2G1; R2U2G2; R3U3G1; R3U3G2; R3U3G3; R3U3G4). Fragmentation of R2U2G2 by MS/MS showed that the two Rha units were branched by one single terminal Gal. No isomers could be observed. These results suggest that the other oligosaccharides (R2U2G1; R2U2G2; R3U3G1; R3U3G2; R3U3G3) could be branched in the same way. Similar oligosaccharides arising from a rhamnogalacturonan hydrolase digestion of deesterified apple modified-hairy regions (Schols *et al.*, 1990) were first identified in NMR by Colquhoun *et al.* 1990. The study was extended to modified-hairy regions from carrot, onion, leek, potato, and pear (Schols *et al.*, 1994). After deesterification and rhamnogalacturonan hydrolase treatment, peaks eluting at the same retention times than peaks found in apple were detected by HPAEC (Schols *et al.*, 1994). The authors suggested that the same fragments occurred in all these plant sources. R2U2G2 oligomers, in which the two Rha units were branched by one single terminal Gal were also isolated from sugar beet RGI (Ralet *et al.*, 2010). In the present study, R3U3G4 was also identified. The extra Gal unit could either be linked on a Rha residue as a second substitution at *O*-3 or be connected to another Gal, most likely at *O*-4. Interestingly, RGI

oligosaccharides carrying both Ara- and Gal-containing side chains were identified (R2U2G2A2; R3U3G2A1; R3U3G2A2). Fragmentation of R2U2G2A2 by MS/MS, revealed that the two Ara units constitute a disaccharide that is linked onto a Gal residue, itself attached at *O*-4 to the internal Rha unit of the DP4 RU backbone. The non-reducing terminal Rha was branched at *O*-4 by a single Gal residue. The exact linkage type between the Ara dimer and the Gal unit could however not be determined. The presence of a R2U2G2A2 oligosaccharide was previously shown in *Gossypium hirsutum* L (Zheng and Mort, 2008). Similarly to our findings, each Rha unit from the DP4 RU backbone was branched with a single Gal residue. In contrast to our findings, single independent Ara*f* residues were found attached at *O*-3 and *O*-4 of one of the two Gal units.

III.4.b RUP2 antibody putative epitope is a peculiar arabinogalactan side chain

Monoclonal antibodies were raised against RUP oligosaccharides and one of them (RUP2 antibody) was characterized in details. Thanks to glycan microarray, it was shown that RUP2 had an original binding pattern compared to the available RGI-related mAbs. RUP2 antibody appeared very specific. It did bind to potato and soybean polymeric RGI and to RUP oligosaccharides and ELISA competitive inhibition assays provided evidence that the epitope was also present in two other galactan-rich plant sources, carrot and lupin. RUP2 however does not react with any of the polymeric and oligomeric pectin-related substrates tested; notably, HG, AGII and oligosaccharides arising from (1→4)-linked galactan, (1→5)-linked arabinan, unbranched RGI backbone and RGI backbone branched by single Gal residues (R2U2G2) were not recognized.

In order to get further insight into the epitope structure, a variant to the epitope detection chromatography technique developed by Cornuault *et al*., (2014) was implemented, in which anion-exchange chromatography-resolved fractions were used as competitors to total RUP oligosaccharides in a competitive inhibition ELISA assay. A neutral very minor fraction consisting in terminal Gal*p*, 1,5-Ara*f* and 1,4-Gal*p* was highly bound by the RUP2 antibody. This implies that the epitope consists in a structurally complex side chain and that the RGI backbone is not involved in epitope recognition. In a previous study, the structural complexity of potato RGI side chains was highlighted (ØBro *et al.*, 2009). Øbro and co-

workers indeed showed that β-(1→4)-galactans were released by endo-arabinanase treatment of purified potato RGI. Furthermore, Ara was released by endo-galactanase treatment of purified potato RGI. The authors suggested that (i) β-(1→4)-galactans may carry few substitutions of short arabinan chains, (ii) α-(1→5)-arabinans may be attached to the RGI backbone through short galactan anchor chains, (iii) β-(1→4)-galactans may be present as side chains or extensions of the arabinans. Complex Ara- and Gal-containing side chains were also evidenced in soybean (Huisman *et al.*, 2001). Indeed, oligosaccharides containing (1→4)-linked Gal*p* residues bearing one Ara*p* residue at the non-reducing end and oligosaccharides made of (1→4)-linked Gal*p* residues interspersed with one internal (1→5)-linked Ara*f* residue were evidenced.

In the present study, oligosaccharides made of (1→4)-linked Gal*p* residues interspersed with internal (1→5)-linked Ara*f* residues were also evidenced. Based on linkage analysis, an average DP of 2 may be estimated for the internal (1→5)-linked-arabinan stretch. α-(1→5)-arabinan dimers attached to the RGI backbone through a single Gal anchor chain were also evidenced, in agreement with Øbro and co-workers hypothesis (ØBro *et al.*, 2009). Interestingly, the anion-exchange chromatography-resolved fraction containing oligosaccharides made of (1→4)-linked Gal*p* residues interspersed with internal (1→5)-linked Ara*f* residues was highly recognized by the RUP2 antibody while the fraction containing α-(1→5)-arabinan dimers attached to a DP4 RGI backbone through a single Gal anchor was not. The RUP2 antibody is therefore likely to be highly specific to the following oligosaccharides:

$[(1\rightarrow4)\text{-}\beta\text{-D-Gal}p]_x\text{-}[(1\rightarrow5)\text{-}\alpha\text{-L-Ara}f]_y\text{-}[(1\rightarrow4)\text{-}\beta\text{-D-Gal}p]_z$

with x, y and z values ranging from1 to 3.

III.4.c Occurrence of the epitope in plant cell wall

While in biochemistry, the epitope of RUP2 has been described to be abundant in potato tuber, prior treatments were required on potato periderm section for its revelation. RUP2, that binds to a complex Ara- and Gal-containing side chain in which (1→4)-linked Gal*p*

residues are interspersed with internal (1→5)-linked Ara*f* residues, was used as a probe to localize this peculiar RGI side chain *in planta*. Immunolabeling of potato tuber sections showed that preliminary enzymatic treatments are necessary to reveal the epitope. Labeling was revealed after endo-galactanase treatment and enhanced by a further treatment with endo-arabinanase. It could be hypothesized that the epitope is at least partly present close to the RU backbone. Indeed, if the short internal (1→5)-linked-arabinose units would have been solely present far from the RU backbone (i.e. long galactan anchor to RU backbone), the galactan anchor would have been hydrolyzed by endo-galactanase and the epitope lost. It is noteworthy that total depletion of potato RGI side chains by extensive arabinan- and galactan-degrading enzymes cannot be achieved (Sørensen *et al.*, 2000; Skjot *et al.*, 2002; O'Bro *et al.*, 2009; Larsen *et al.*, 2011). Structures recalcitrant to enzymatic degradation are likely to encompass RUP2 antibody epitope.

In agreement with previous studies (Bush *et al.*, 2001; Oomen *et al.*, 2002), arabinan and galactan in potato tuber were found abundant in the primary wall and absent from the expanded middle lamellae at cell corners. Contrary to Lee *et al*. (2014), INRA-RU1 epitope was detected in both primary cell wall and middle lamella. This apparent discrepancy could be because in the study of Lee *et al*. (2014), tobacco seed endosperm cross sections were not pre-treated with RGI side chain degrading enzymes prior to immunolabeling. It can be speculated that the unbranched region of the RGI backbone in primary cell wall was masked by the presence of side chains. Surprisingly, RUP2 epitope was found to be restricted to middle lamella and cell corners whatever the pre-treatment prior to immunolabeling. In *Arabidopsis thaliana* seedling roots, RUP2 epitope was localized in the elongation zone and no galactanase pre-treatment was required. These results are preliminary and the occurrence of RUP2 epitope has to be checked at the ultrastructural level using TEM and investigated in other plant species.

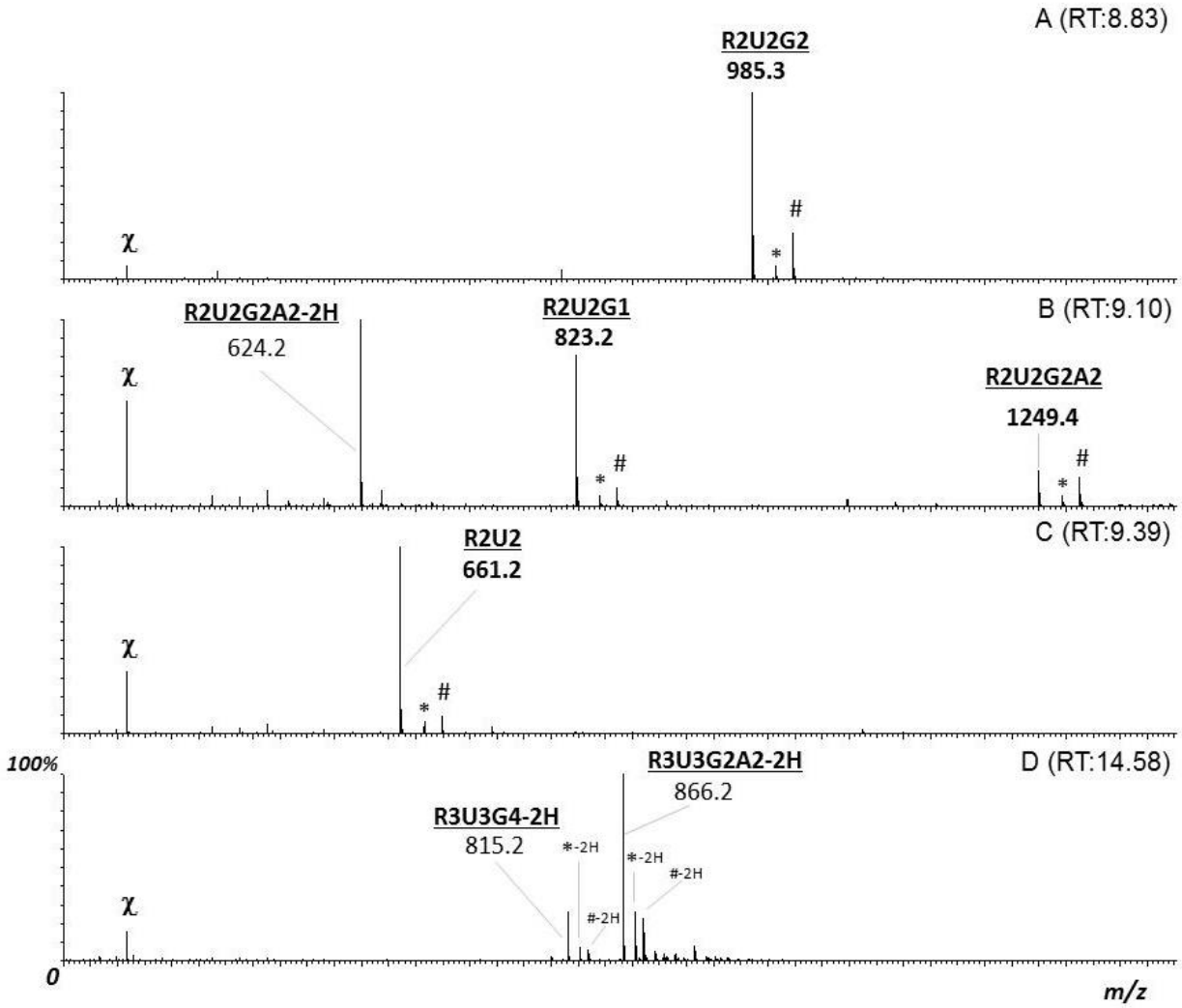
These results as others highlight the heterogeneity of the distribution pattern of the different pectin domains at the cell wall level and the differential masking of epitopes across cell walls and middle lamellae. They raise questions about the arrangement of these domains within the pectic molecule, their integration into the cell wall and their configuration within the wall. Clearly, further work is required for understanding RGI glycan

complexity in the context of cell-wall architecture and in relation to cell-wall functions in plant life.

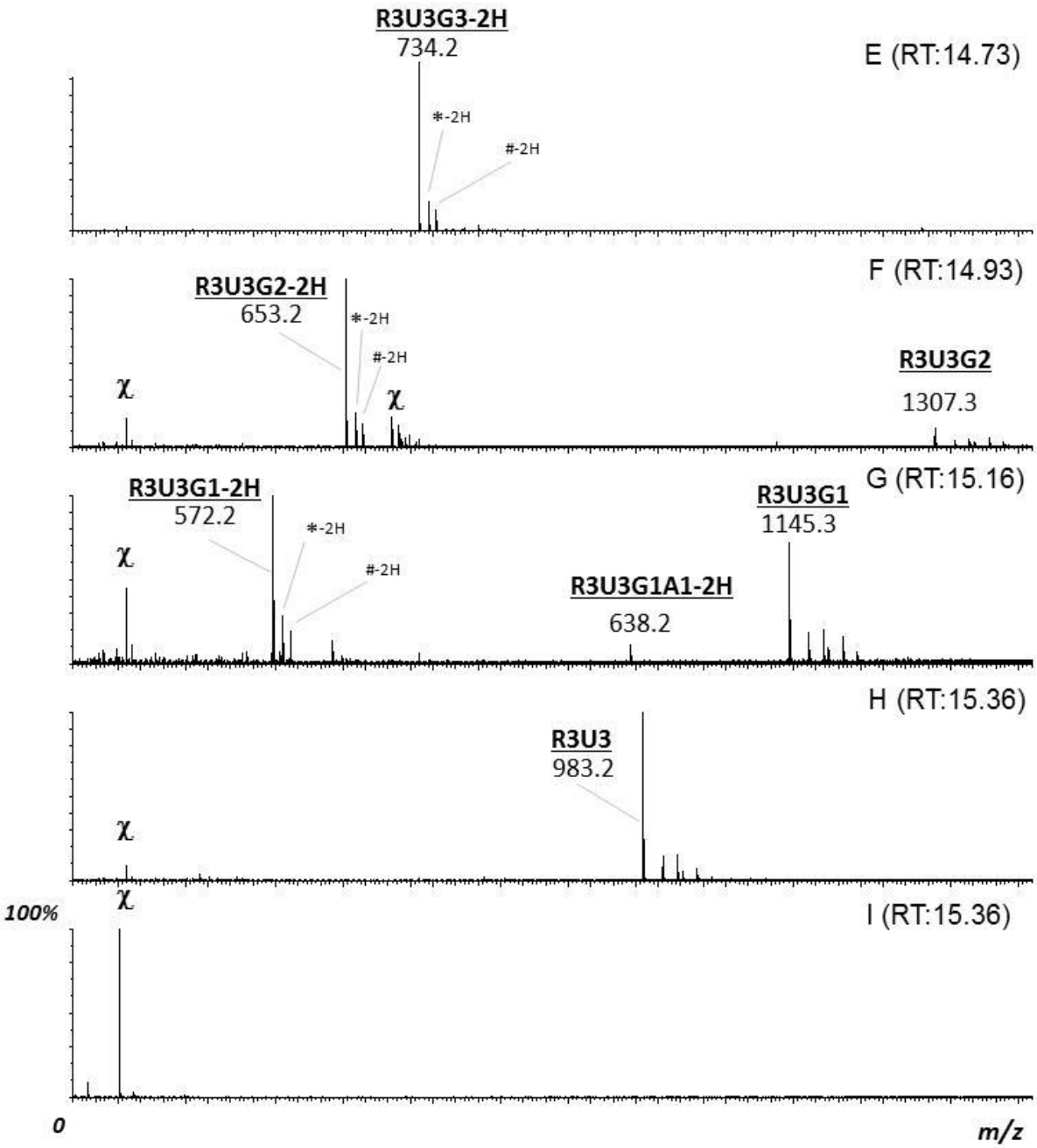
III.5 Conclusion

RUP2 is a valuable addition to the set of antibodies that can detect various pectic structural domains within the cell walls. It can provide new insight into pectin structure and processing in plant cell walls in relation to cell biological events and functions.

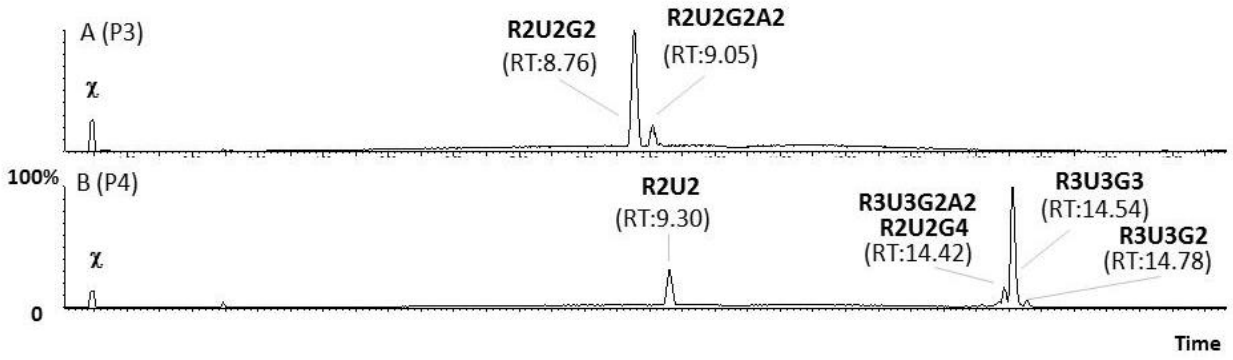
Supplementary datas



Supplementary figure III.1: Detailed spectra of the pics A, B, C, D detected on LC-MS (CF: Figure.III.5) from RUP oligosaccharides. χ : are contaminants. *: Na adducts. #: K adducts. RT: retention time of the pic considered.



Supplementary figure III.2: Detailed spectra of the peakcs E, F, G, H, I detected on LC-MS (CF: Figure.III.5) from the RUP oligosaccharides. χ : are contaminants. *: Na adducts. #: K adducts. RT: retention time of the pic considered.



Supplementary figure III.3: Separation of oligosaccharides present in each pool (P1-5) on LC-MS (CF:Figure.III.4). χ : are contaminants. R: Rha ; U: Galacturonic acid; G: galactose; A: arabinose; RT: retention time of the pic considered.

References

Albersheim P, Darvill AG, O'Neill MA, Schols HA, Voragen AGJ. 1996. An Hypothesis: The same six polysaccharides are components of the primary cell wall of all higher plants. In J. Visser, & A. G. J.Voragen. *Pectins and Pectinases* Amsterdam (Elsevier): 47-53.

Anumula KR, Taylor PB. 1992. A comprehensive procedure for preparation of partially methylated alditol acetates from glycoprotein carbohydrates. *Analytical Biochemistry* **203**(1): 101-108.

Bonnin E, Dolo E, Le Goff A, Thibault J-F. 2002. Characterisation of pectin subunits released by an optimised combination of enzymes. *Carbohydrate Research* **337**(18): 1687-1696.

Bradford MMA. 1976. a rapid and sensitive method for the quantification of the protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-255.

Branca C, Lorenzo GD, Cervone F. 1988. Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. *Physiologia Plantarum* **72**(3): 499-504.

Buckeridge MS, Hutcheon IS, Reid JS. 2005. The role of exo-(1-->4)-beta-galactanase in the mobilization of polysaccharides from the cotyledon cell walls of Lupinus angustifolius following germination. *Annals of Botany* **96**(3): 435-444.

Bush MS, Marry M, Huxham IM, Jarvis MC, McCann MC. 2001. Developmental regulation of pectic epitopes during potato tuberisation. *Planta* **213**(6): 869-880.

Bush MS, McCann MC. 1999. Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiologia Plantarum* **107**(2): 201-213.

Caffall KH, Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. *Carbohydrate research* **344**(14): 1879-1900.

Carpita NC, Gibeaut DM. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant journal for cell and molecular biology* **3**(1): 1-30.

Chylinska M, Szymanska-Chargot M, Zdunek A. 2014. Imaging of polysaccharides in the tomato cell wall with Raman microspectroscopy. *Plant Methods* **10**(14).

Clausen MH, Willats WG, Knox JP. 2003. Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan monoclonal antibodies LM7, JIM5 and JIM7. *Carbohydrate Research* **338**(17): 1797-1800.

Cornuault V, Manfield IW, Ralet MC, Knox JP. 2014. Epitope detection chromatography: a method to dissect the structural heterogeneity and inter-connections of plant cell-wall matrix glycans. *Plant Journal* **78**(4): 715-722.

Domon B, Costello CE. 1988. A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. *Glycoconjugate Journal* **5**(4): 397–409.

Englyst HN, Cummings JH. 1988. Improved method for measurement of dietary fiber as non-starch polysaccharides in plant foods. *journal association of analytical chemistry* **71**(4): 808-814.

Gomez LD, Steele-King CG, Jones L, Foster JM, Vuttipongchaikij S, McQueen-Mason SJ. 2009. Arabinan metabolism during seed development and germination in Arabidopsis. *Molecular Plant* **2**(5): 966-976.

Ha MA, Vietor RJ, Jardine GD, Apperley DC, Jarvis MC. 2005. Conformation and mobility of the arabinan and galactan side-chains of pectin. *Phytochemistry* **66**(15): 1817-1824.

Huisman MM, Brul LP, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AG. 2001. The occurrence of internal (1 --> 5)-linked arabinofuranose and arabinopyranose residues in arabinogalactan side chains from soybean pectic substances. *Carbohydrate Research* **330**(1): 103-114.

Ishii T. 1997. O-acetylated oligosaccharides from pectins of potato tuber cell walls. *Plant Physiology* **113**(4): 1265-1272.

Ishii T, Matsunaga T. 2001. Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry* **57**(6): 969-974.

Jarvis MC, Hall MA, Threlfall DR, Friend J. 1981. The polysaccharide structure of potato cell walls: Chemical fractionation. *Planta* **152**(2): 93-100.

Jones L, Seymour GB, Knox JP. 1997. Localization of Pectic Galactan in Tomato Cell Walls Using a Monoclonal Antibody Specific to (1-->4)-[beta]-D-Galactan. *Plant physiology* **113**(4): 1405-1412.

Jones L, Milne JL, Ashford D, McCann MC, McQueen-Mason SJ. 2005. A conserved functional role of pectic polymers in stomatal guard cells from a range of plant species. *Planta* **221**(2): 255-264.

Khodaei N, Karboune S. 2013. Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall. *Food Chemistry* **139**(1-4): 617-623.

Koornneef M, Meinke D. 2010. The development of Arabidopsis as a model plant. *Plant J* **61**(6): 909-921.

Larsen FH, Byg I, Damager I, Diaz J, Engelsen SB, Ulvskov P. 2011. Residue specific hydration of primary cell wall potato pectin identified by solid-state 13C single-pulse MAS and CP/MAS NMR spectroscopy. *Biomacromolecules* **12**(5): 1844-1850.

Lau JM, McNeil M, Darvill AG, Albersheim P. 1987. Treatment of rhamnogalacturonan I with lithium in ethylenediamine. *Carbohydrate Research* **168**(2): 245-274.

Le Goff A, Renard CMGC, Bonnin E, Thibault JF. 2001. Extraction, purification and chemical characterisation of xylogalacturonans from pea hulls. *Carbohydrate Polymers* **45**(4): 325-334.

Lerouge P, O'Neill MA, Darvill AG, Albersheim P. 1993. Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydrate Research* **243**(2): 359-371.

Marcus SE, Blake AW, Benians TA, Lee KJ, Poyser C, Donaldson L, Leroux O, Rogowski A, Petersen HL, Boraston A, Gilbert HJ, Willats WG, Knox JP. 2010. Restricted access of proteins to mannan polysaccharides in intact plant cell walls. *Plant J* **64**(2): 191-203.

Marfà V, Gollin DJ, Eberhard S, Mohnen D, Dan/ill A, Albersheim P. 1991. Oligogalacturonides are able to induce flowers to form on tobacco explants. *The Plant Journal* **1**(2): 217-225.

Massiot P, Rouau X, Thibault JF. 1988. Structural study of the cell-wall of carrot (daucus-carota l) .1. isolation and characterization of the cell-wall fibers of carrot. *Carbohydrate Research* **172**(2): 217-227.

Mazurek S, Mucciolo A, Humbel BM, Nawrath C. 2013. Transmission Fourier transform infrared microspectroscopy allows simultaneous assessment of cutin and cell-wall polysaccharides of Arabidopsis petals. *Plant Journal* **74**(5): 880-891.

Moller I, Marcus SE, Haeger A, Verhertbruggen Y, Verhoef R, Schols H, Ulvskov P, Mikkelsen JD, Knox JP, Willats W. 2008. High-throughput screening of monoclonal antibodies against plant cell wall glycans by hierarchical clustering of their carbohydrate microarray binding profiles. *Glycoconjugate Journal* **25**(1): 37-48.

Mouille G, Robin S, Lecomte M, Pagant S, Hofte H. 2003. Classification and identification of Arabidopsis cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *Plant Journal* **35**(3): 393-404.

O'Bro J, Borkhardt B, Harholt J, Skjot M, Willats WG, Ulvskov P. 2009. Simultaneous in vivo truncation of pectic side chains. *Transgenic Res* **18**(6): 961-969.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG. 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* **294**(5543): 846-849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology* **55**: 109-139.

ØBro J, Borkhardt B, Harholt J, Skjot M, Willats WG, Ulvskov P. 2009. Simultaneous in vivo truncation of pectic side chains. *Transgenic Research* **18**(6): 961-969.

Oomen RJFJ, Doeswijk-Voragen CHL, Bush MS, Vincken J-P, Borkhardt B, Van Den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF. 2002. In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. *The Plant Journal* **30**(4): 403-413.

Ovodov YS, Ovodova RG, Bondarenko OD, Krasikova IN. 1971. The pectic substances of zosteraceae : Part IV. Pectinase digestion of zosterine. *Carbohydrate Research* **18**(2): 311-318.

Pedersen HL, Fangel JU, McCleary B, Ruzanski C, Rydahl MG, Ralet MC, Farkas V, von Schantz L, Marcus SE, Andersen MC, Field R, Ohlin M, Knox JP, Clausen MH, Willats WG. 2012. Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research. *Journal of Biological Chemistry* **287**(47): 39429-39438.

Proseus TE, Boyer JS. 2006. Calcium pectate chemistry controls growth rate of Chara corallina. *Journal of Experimental Botany* **57**(15): 3989-4002.

Ralet MC, Dronnet V, Buchholt HC, Thibault JF. 2001. Enzymatically and chemically de-esterified lime pectins: characterisation, polyelectrolyte behaviour and calcium binding properties. *Carbohydrate Research,* **336**: 117-25.

Ralet MC, Crépeau MJ, Buchholt HC, Thibault JF. 2003. Polyelectrolyte behaviour and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. *Biochemical Engineering Journal,* **16**: 191-201.

Ralet MC, Tranquet O, Poulain D, Moise A, Guillon F. 2010. Monoclonal antibodies to rhamnogalacturonan I backbone. *Planta* **231**(6): 1373-1383.

Renard CM, Crepeau MJ, Thibault JF. 1999. Glucuronic acid directly linked to galacturonic acid in the rhamnogalacturonan backbone of beet pectins. *European Journal of Biochemistry* **266**(2): 566-574.

Ridley BL, O'Neill MA, Mohnen DA. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* **57**(6): 929-967.

Roy R, Katzenellenbogen E, Jennings HJ. 1984. Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. *Canadian Journal of Biochemistry and Cell Biology* **62**(5): 270-275.

Sabba RP, Lulai EC. 2004. Immunocytological comparison native and wound periderm maturation in potato tuber. *American Journal of Potato Research* **81**(2): 119-124.

Schols HA, Geraeds CCJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ. 1990. Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. *Carbohydrate Research* **206**(1): 105-115.

Schols HA, Voragen AG, Colquhoun IJ. 1994. Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. *Carbohydr Research* **256**(1): 97-111.

Schols HA, Voragen AGJ. 1994. Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. **256**(1): 83-95.

Shibuya N, Minami E. 2001. Oligosaccharide signalling for defence responses in plant. *Physiological and Molecular Plant Pathology* **59**(5): 223-233.

Skjot M, Pauly M, Bush MS, Borkhardt B, McCann MC, Ulvskov P. 2002. Direct interference with rhamnogalacturonan I biosynthesis in Golgi vesicles. *Plant Physiology* **129**(1): 95-102.

Sørensen I, Pedersen HL, Willats WGT. 2009. An array of possibilities for pectin. *Carbohydrate Research* **344**(14): 1872-1878.

Sørensen SO, Pauly M, Bush M, Skjot M, McCann MC, Borkhardt B, Ulvskov P. 2000. Pectin engineering: Modification of potato pectin by in vivo expression of an endo-1,4-beta-D-galactanase. *Proceedings of the National Academy of Sciences of the United States of America* **97**(13): 7639-7644.

Tang H, Belton PS, Ng A, Ryden P. 1999. 13C MAS NMR studies of the effects of hydration on the cell walls of potatoes and Chinese water chestnuts. *Journal of Agricultural and Food Chemistry* **47**(2): 510-517.

Thibault JF. 1979. Automatisation du dosage des substances pectiques par la methode au méta-hydroxydiphényle. *Lebensmittel-Wissenschaft und-Technologie* **71**: 215-223.

Tollier MT, Robin JP. 1979. Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. *Annales de Technologie Agricole* **28**: 1-15.

Verhertbruggen Y, Marcus SE, Haeger A, Ordaz-Ortiz JJ, Knox JP. 2009a. An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydrate Research* **344**(14): 1858-1862.

Verhertbruggen Y, Marcus SE, Haeger A, Verhoef R, Schols HA, McCleary BV, McKee L, Gilbert HJ, Knox JP. 2009b. Developmental complexity of arabinan polysaccharides and their processing in plant cell walls. *Plant Journal* **59**(3): 413-425.

Willats WGT, Marcus SE, Knox JP. 1998. Generation of a monoclonal antibody specific to (1-->5)-α-L-arabinan. *Carbohydrate Research* **308**(1-2): 149-152.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001. Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology* **47**(1): 9-27.

Willats WGT, McCartney L, Steele-King C, Marcus S, Mort A, Huisman M, Alebeek G-J, Schols H, Voragen AJ, Goff A, Bonnin E, Thibault J-F, Knox JP. 2004. A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta* **218**(4): 673-681.

Young RE, McFarlane HE, Hahn MG, Western TL, Haughn GW, Samuels AL. 2008. Analysis of the Golgi apparatus in Arabidopsis seed coat cells during polarized secretion of pectin-rich mucilage. *Plant Cell* **20**(6): 1623-1638.

Zheng Y, Mort A. 2008. Isolation and structural characterization of a novel oligosaccharide from the rhamnogalacturonan of Gossypium hirsutum L. *Carbohydrate Research* **343**(6): 1041-1049.

Zykwinska A, Rondeau-Mouro C, Garnier C, Thibault JF, Ralet MC. 2006. Alkaline extractability of pectic arabinan and galactan and their mobility in sugar beet and potato cell walls. *Carbohydrate Polymers* **65**(4): 510-520.

CHAPTER IV

General discussion

Establishing a relationship between a polysaccharide's fine structure and its function *in planta* is an enormous challenge, especially when the polysaccharide is highly heterogeneous. RGI and RGII are both very complex pectic domains, exhibiting a large structural versatility. Evaluation of the structure variability *in planta* is the first step for understanding the function related to a specific structure. *In situ*, microscopy with the use of mAbs, is a technique which already allowed to localize several pectic structural domains with a good resolution. Useful mAbs against rhamnogalacturonans are available but they do not represent the polymers complexity. Production of new mAbs is a difficult task since many parameters cannot be fully controlled. Also, when new mAbs are successfully obtained, the epitope recognized might not be what was targeted at the beginning of the study. However, the use of new mAbs on plant tissues would always provide information about the distribution of the detected epitope. In this work, RGI and RGII motifs were isolated for the production of new mAbs. Issues met at all different steps from the isolation and purification of RGI and RGII motifs to the use of the produced mAbs are discussed in this chapter and some research perspectives are highlighted.

IV.1 Challenges for rhamnogalacturonans structural characterization

HG, the simplest and the most abundant pectic domain, has been widely studied. HG domains are indeed responsible for pectin gelling properties (Willats *et al.*, 2006). Today, for many reasons, other pectic domains are gaining more and more interest. In food and beverage products, RGI and RGII have been shown to impact on the process efficiency and the qualitative aspects of the final product (Schols *et al.*, 1990; Willats *et al.*, 2006; Ducasse *et al.*, 2011). Identification of β-1,4-galactan synthase was also suggested to be an interesting tool for engineering plants with improved bioenergy properties (Liwanag *et al.*, 2012). RGI oligosaccharides have also been largely described as possible health promoting agents in animal and human (Gao *et al.*, 2013; Leclere *et al.*, 2013). Because of all these applications, an increasing number of studies focus on RGI and RGII fine structural characterization. In the present work, we experienced that the purification and structural characterization of rhamnogalacturonans remain a complex and difficult task.

IV.1.a Extraction of rhamnogalacturonans and residual contaminants issue

Even though RGII accounts for 1 to 4% of the eudicotyledon walls only (O'Neill *et al.*, 2004), it has an essential physiological role *in planta* (O'Neill *et al.*, 2001). In depth structural characterization of this pectic domain was mainly conducted on wine RGII. Indeed, despite polyphenol and microbial polysaccharides have to be removed, wine is a very good source of RGII, which represents up to 20% of wine total polysaccharides (Doco and Brillouet, 1993; Pellerin *et al.*, 1996; Vidal *et al.*, 2000; Vidal *et al.*, 2003). The extraction and purification process of wine RGII is now well established as fully described in chapter II. In contrast, improvement of RGI domain extraction and purification is still an issue (Khodaei and Karboune, 2013).

Pectin can be recovered using different extraction methods. Chelation of cations by EDTA or CDTA enables pectin solubilization. However, the recovery yield is rather low (Renard and Thibault, 1993). In addition, the difficulty to remove the chelating agent has to be

considered. For a better pectin extractability, acid treatment is preferably used (Levigne *et al.*, 2004). Yet, degradation of side chains, especially arabinans, occurs (Schols *et al.*, 1990). Under alkaline conditions, pectin can be extracted with good recovery yield and side chains are well preserved (Bonnin *et al.*, 2001; Zykwinska *et al.*, 2006; Khodaei and Karboune, 2013). Alkali conditions are however propitious to pectin deesterification and information about methyl- and acetyl-esterification location is lost. Using harsh alkaline conditions, as described in chapter III, HGs are degraded by beta-elimination, releasing the RGI domains. For RGI recovery, another method consists in the degradation of HGs by enzymatic treatment. Extraction of RGI with endo-polygalacturonase has been widely used. To allow the enzymatic reaction, prior de-esterification has to be performed (ØBro *et al.*, 2004; Khodaei and Karboune, 2013). Compared to alkaline extraction, this method allows the recovery of RGI of higher molar mass (Khodaei and Karboune, 2013). Moreover, the enzymatic method leads to a lower molar proportion of residual sugars such as Glc, Xyl and Man than the alkaline one (Khodaei and Karboune, 2013). This would explain the higher amount of these sugars in our potato NaOH-extracted RGI (chapter III), compared to the potato endo-polygalacturonase-extracted RGI described by Øbro *et al*. (2004). These sugars may either be part of the RGI side chains or part of distinct co-extracted cell wall components, such as glycoproteins and hemicelluloses. AGPs and hemicelluloses such as xylan and xyloglucan were described to be potentially branched to RGI (Nakamura *et al.*, 2002; Popper and Fry, 2008; Zaidel and Meyer, 2012; Tan *et al.*, 2013). The work of Cornault *et al.* (2014) provided evidence that glucuronoxylan are present in the RUP oligosaccharides extracted from potato RGI. This study further highlighted that glucuronoxylan motifs may be covalently linked to RGI.

IV.1.b Production of oligosaccharides is essential for structural characterization

Pectin is an extremely complex multiblock biopolymer and global analyses on the whole macromolecule are not sufficient to give insight into the pectin fine structure. Therefore, pectin is commonly degraded into oligosaccharides by chemical and/or enzymatic means.

After fractionation of the degradation products, isolated structural elements, which are in the analytical range of a broad set of techniques, can be obtained (Ralet *et al.*, 2009).

Regarding RGII, only a soil fungus was reported to be able to degrade the branched RGII backbone into two pieces, indicating that *Penicillium daleae* is a potential source for new pectinase (Vidal *et al.*, 1999). Therefore, RGII glycosyl sequence was mostly determined after chemical fragmentation of the molecule and partial degradation by mild acid hydrolysis – usually with trifluoroacetic acid (TFA) – has been the most widely used method (Spellman *et al.*, 1983; Stevenson *et al.*, 1988; Thomas *et al.*, 1989; Whitcombe *et al.*, 1995; Shin *et al.*, 1998; Matsunaga *et al.*, 2004; Reuhs *et al.*, 2004; Séveno *et al.*, 2009; Voxeur *et al.*, 2011; Pabst *et al.*, 2013).

In contrast to RGII, a large set of enzymes is available for the release of RGI fragments (Bonnin *et al.*, 2014). Esterases and depolymerases, are the two different kinds of enzymes used for RGI degradation. In chapter III, the purified potato RGI was digested by endo-arabinanase and endo-galactanase to remove linear side chains of arabinan and galactan, respectively. The low-branched RGI backbone could then be depolymerized using a rhamnogalacturonan hydrolase. From all these successive enzymatic reactions, RUP oligosaccharides were successfully recovered. However, a recalcitrant fraction, which was separated from the RUP oligosaccharides by ethanol precipitation, was still present. Schols and co-workers faced the same problem when isolating RGI-related oligosaccharides from modified hairy regions (MHR) obtained by enzymatic treatment of different cell wall materials (Schols *et al.*, 1990). Apple MHR was prepared using an enzymatic mixture (Rapidase C600) and further depolymerized by a rhamnogalacturonan hydrolase. It is noteworthy that rhamnogalacturonan hydrolase was not active against MHR but very active towards deesterified MHR. It was also active towards HCl-treated-MHR that was depleted in arabinan but also largely deacetylated. After fractionation by size-exclusion chromatography of a rhamnogalacturonan hydrolase digest of HCl-treated MHR, RGI oligomers were successfully isolated. However a large amount of high molecular weight material was still present in the digest. This recalcitrant fraction encompassed xylogalacturonan polymers and presumably RGII but also non-degradable Gal-rich RGI large oligomers. Both studies (Schols and co-workers one and the present one) illustrate that enzymatic tools are still lacking for a complete degradation of the complex RGI structure.

IV.1.c Structural characterization of RGI fragments

NMR has been widely used for structural characterization of cell-wall-derived oligosaccharides. The main drawbacks of NMR techniques are, however, sample purity and concentration requirements. The high sensitivity, high throughput and capacity to analyze mixtures of the new generation of mass analyzers led to a re-emergence of the use of mass spectrometry for oligosaccharides structural characterization in the past 15 years (Ralet *et al.*, 2009).

In the present work, MS was shown to be a powerful technique that allowed an in-depth description of RGII side chain A and side chain B structural variation (chapter II). RUP oligosaccharides were also characterized by mass spectrometry (chapter III). The detected oligosaccharides systematically encompassed a DP 4 or DP 6 RGI backbone. Beside limited amounts of unbranched oligosaccharides (R2U2; R3U3), most of the oligosaccharides detected contained Gal residues only (R2U2G1; R2U2G2; R3U3G1; R3U3G2; R3U3G3; R3U3G4). Fragmentation of R2U2G2 by MS/MS showed that the two Rha units were each branched by a single terminal Gal. The presence of high proportions of terminally-linked Gal*p* residues in apple MHR has already been discussed (Schols, 1995). By studying the rhamnogalacturonan digests of apple (non-modified) hairy regions, Schols (1995) came to the conclusion that native pectin comprises RGI containing a rather high proportion of Rha*p* units branched by single Gal*p* residues. In the present work, RGI oligosaccharides carrying both Ara- and Gal-containing side chains were also identified (R2U2G2A2; R3U3G2A1; R3U3G2A2). Previous structural studies reported that β-D-Gal*p* and/or α-L-Ara*f* residues could be substituted on the α-L-Rha units (Table.IV.1). However, substitution by β-D-Gal*p* units has been much more commonly described whatever the RGI source, arabinan- or galactan-rich (Gur'janov *et al.*, 2007; Ralet *et al.*, 2010). Only few studies reported direct substitution of α-L-Ara*f* onto Rha*p* units (Lau *et al.*, 1987; Ducasse *et al.*, 2011).

Table IV.1 : Fragments illustrating RGI structural complexity

Structure	Plant source	Reference
β-D-Gal*p*-(1→4)-L-Rhaol β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-L-Rhaol β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol α-L-Fuc*f*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-L-Rhaol Ara-(1→4)-L-Rhaol Ara-Ara-Rhaol Fuc-Ara-Rhaol α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol α-L-Ara*f*-(1→2)-α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol α-L-Ara*f*-(1→5)α-L-Ara*f*-(1→2)-α-L-Ara*f*-(1→3)-β-D-Gal*p*-(1→4)-L-Rhaol	Suspension-cultured sycamore cells	Lau *et al.,* 1987
-Rha*p* 1→4 α-Gal*p*A 1→2 α-Rha*p* 1 →4 α-Gal*p*A1 4 4 ↑ ↑ 1 1 β-Gal*p* β-Gal*p*	Apple	Colquhoun *et al.*, 1990
Rha*p* 1→4 α-Gal*p*A 1→2 α-Rha*p* 1 →4 α-Gal*p*A 1→2 Rha*p* 1→4 α-Gal*p*A 4 4 4 ↑ ↑ ↑ 1 1 1 β-Gal*p* β-Gal*p* β-Gal*p*	Apple, carrot, potato, leek, pear, onion	Schools and Voragen, 1994
β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→3)- α/β-D-Gal*p*-OH→ attached to arabinogalactan type I backbone	Potato, soybean, citrus, onion	Hinz *et al.*, 2005
α-Rha*p* 1→4 α-Gal*p*A 1→2 α-Rha*p* 1 →4 α-Gal*p*A1 4 4 ↑ ↑ 1 1 β-Gal*p* α-Ara*f* 1 →3 β-Gal*p* 4 ←1 α-Ara*f*	*Gossypium hirsutum* L.	Zeng *et al.*, 2008
Internal (1→5)-α-L-Araf residue in a DP5 chain of (1→4)-β-D-Gal*p*	Soybean	Huisman *et al.*, 2001
α-L-Ara*f*-(1→3)α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5) -α-L-Ara*f* 3 ↑ 1 α-L-Ara*f* α-L-Ara*f*-(1→3)α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f* 3 2 3 ↗ ↖ ↑ 1 1 1 α-L-Ara*f* α-L-Ara*f* α-L-Ara*f* α-L-Ara*f*-(1→3)α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→5) -α-L-Ara*f* 3 3 ↑ ↑ 1 1 α-L-Ara*f* α-L-Ara*f*	Sugar beet	Westphal *et al.*, 2010
GalA-(Rha-Ara-Ara) Rha-GalA-(Rha-Gal) Rha-GalA-Rha-GalA	Wine	Ducasse *et al.*, 2011
β-D-Glc*p*-(1→6)-β-D-Galp_{sc}-(1→ α-D-Gal*p*A-(1→2)-α-L-Rha-(1→4)-β-D-Glc*p*A β-Xyl*p*-(1→4)-α-L-Rha*p*	A.*thaliana*	Tan *et al.*, 2013

This observation may be the consequence of two phenomena: direct branching of α-L-Ara*f* onto the Rha*p* units is rare or/and this branching is more likely present in the recalcitrant fraction. On RUP oligosaccharides no α-L-Ara*f* residues have been detected branched on the Rha*p* units. Ara- and Gal-containing oligosaccharides were seen to contain α-L-Ara*f* disaccharides indirectly branched on a Rha*p* unit through a β-D-Gal*p* residue (chapter III). Such a structure has already been described in several studies (Lau *et al.*, 1987; Zheng and Mort, 2008) indicating that arabinan side chains are likely to be initiated by a β-D-Gal*p* unit.

Full structural characterization of RGI remains a difficult task. Indeed, over 15 different linkages are constitutive of the polymer, some of them being very minor. Isolating minor atypical oligosaccharides that are 'buried' in large amounts of 'classical' ones is very challenging. The different structures listed on Table IV.1 give an idea of the real complexity of RGI side chains.

IV.2 Production of mAbs

RGI and RGII fragments were the motifs targeted for mAbs production. No mAb to RGII are available and RGII localization in cell walls has only been achieved using polyclonal antibodies (Matoh *et al.*, 1998). In contrast to monoclonal antibodies, polyclonals are not specific to one unique antigen and are not inexhaustible. Therefore, mAbs are preferred for a precise recognition of a peculiar structure. In this project, we chose to isolate RGII side chain A and side chain B. Indeed, the Gal*p*A backbone is suspected to be an immuno-dominant motif (Verhertbruggen *et al.*, 2009a). Side chain A and side chain B have been detached from the HG backbone by mild acid hydrolysis and purified by anion-exchange chromatography (chapter II). Both side chains have been used for mice immunization.

RGI oligosaccharides from sugar beet (RUB oligosaccharides) and potato (RUP oligosaccharides) were prepared from sugar beet and potato pulp NaOH-extracts, respectively. Both raw RGIs were purified by anion-exchange chromatography prior to partial chemical and/or enzymatic side chains removal. The low-branched RGIs were then hydrolyzed by rhamnogalacturonan hydrolase and recalcitrant fractions were removed by

ethanol precipitation. Mice immunization was performed with RUB and RUP oligosaccharides.

From the four immunizations, mAbs to RUP oligosaccharides only were obtained. Using glycan micro-arrays, the 22 selected clones were found to have similar and very selective patterns of recognition. Namely, only potato and soybean RGIs were recognized. Immunization with RUB and RUP oligosaccharides was also performed at University of Leeds (Valérie Cornuault, Prof. J.P. Knox). Interestingly, results obtained at University of Leeds did not converge with those obtained at INRA-Nantes. mAbs to RUP oligosaccharides were much more diverse and one mAb was shown to recognize glucuronoxylans. In the following chapters, immunizations at University of Leeds and at INRA-Nantes will be confronted and critical points discussed.

IV.2.a Preparation of the glycan immunogen

To generate mAbs, an immunogen has to be prepared. For glycans there are two possibilities, polysaccharides or oligosaccharides injection. Immunizations with polysaccharides are rather easy since the glycan of interest is extracted, eventually purified, and directly injected. Thus, xylogalacturonan (Willats *et al.*, 2004), whole sugar beet pectin (Clausen *et al.*, 2004), whole apple pectin (Verhertbruggen *et al.*, 2009b) and *Arabidopsis thaliana* seeds mucilage (Verhertbruggen *et al.*, 2009a) have been used as immunogens. The fact that the injected polymer encompasses numerous different constitutive motifs may increase the chance to generate new mAbs. However, whole polysaccharides are also likely to contain impurities that could induce immunogenicity. The epitope recognized might not be what was targeted at the beginning of the study. As an example, LM18 and LM20, two mAbs that recognize HG domains have been generated from rats immunized with *Arabidopsis thaliana* mucilage, which is constituted of >90% of unbranched RGI (Verhertbruggen *et al.*, 2009a). Some stretches of HG domains having different degrees of methylesterification are also present in low amounts (Macquet *et al.*, 2007). Interestingly the immune system was launched by the rare HG fragments rather than by the highly concentrated unbranched RGI (Verhertbruggen *et al.*, 2009a). These finding illustrate the immuno-dominance of peculiar structural motifs. Generation of highly purified

oligosaccharides increases probabilities to obtain mAbs recognizing an aimed domain. Nonetheless, although it is a more secure strategy, it generally involves a substantial upstream work. Furthermore, oligosaccharides that are known to be less immunogenic than polysaccharides have to be covalently linked to an immunogenic carrier protein such as BSA or OVA prior to immunization.

IV.2.b Immunization and clone selection

Immunization can be performed using short or long protocols. In the long immunization protocol, hybridomas are generated only if immunoglobulins are detected in animal blood. Immunization-periods can be adapted following the immunogenicity of the glycan injected (Willats *et al.*, 1998; Verhertbruggen *et al.*, 2009a). In short immunization no tails blood is taken to test for the presence of immunoglobulins by ELISA. Therefore, it is not known if lymphocytes of interest are produced by animals before fusion with myeloma cells.

Immunization with RUP and RUB oligosaccharides led to the generation of different mAbs at University of Leeds and at INRA-Nantes. At INRA-Nantes, oligosaccharides were injected in mice using a short immunization protocol whereas at University of Leeds oligosaccharides were injected in rats using a long immunization protocol. Differences observed in mAbs characteristics may be due to the protocol used. However, immunization with side chain A and side chain B were performed at INRA-Nantes using both long and short protocols. In both cases no mAbs recognizing RGII were generated. Therefore, it is likely that other parameters are essential for the production of mAbs. It is suspected that variation in mAbs production and characteristics could be partly due to the choice of the animal. At INRA Nantes 22 clones producing mAbs against RUP oligosaccharides were obtained. However, the 22 hybridomas all produced mAbs that have the same stringent recognition pattern to glycans. At University of Leeds, using the same antigen (RUP oligosaccharides), 4 groups of clones having different patterns of recognition to glycans were recovered (Cornuault *et al.*, 2014). A higher diversity of immune system response may be induced in rats compared to mice.

Differences observed in mAbs characteristics may also be due to the clone selection protocol used. At INRA-Nantes and at University of Leeds, clone selection was performed using ELISA assays. Glycans containing the immunogens are coated onto ELISA plates. Supernatants of clones recognizing these glycans are those selected for further characterization. After RUP immunizations at INRA-Nantes and University of Leeds, clones were not selected using the same glycan. At INRA-Nantes, RUP oligosaccharides conjugated to BSA have been used for clone selection whereas raw RGI from potato was used at University of Leeds. Some motifs, such as glucuronoxylan motifs, are most likely much more represented in raw RGI compared to RUP oligosaccharides. Therefore clone selection at University of Leeds was less stringent than at INRA-Nantes. To avoid missing clones at the selection step, glycan micro-array is a promising screening technique since a very large set of glycans are printed. Furthermore, each glycan can be tested at the same level of concentration (Gro Rydahl, *Unpublish results*).

IV.2.c Epitope characterization

Characterization of mAbs epitopes is usually performed by testing the sensitivity of probes to a large panel of glycans. Indirect ELISA and dotblot have been widely used (Jones *et al.*, 1997; Willats *et al.*, 1998) . Furthermore, enzymatic treatments can be applied on coated glycans prior to the ELISA assay. Signal modification induced by specific enzymes can be evaluated and this can help for the epitope determination (Verhertbruggen *et al.*, 2009b). An extended procedure to indirect ELISA is glycan micro-array, which enables to test a large panel of glycans in one experiment (Sørensen and Willats, 2011; Pedersen *et al.*, 2012). In the present work, RUP antibodies together with some well-known pectin-related mAbs, were probed on a nitrocellulose plate printed with more than 100 different glycans.

Interestingly, some well-known and well-characterized mAbs exhibited unexpected binding. LM6, which binds to linear arabinan showed a quite high avidity to R2U2G2, which does not contain Ara residues (Ralet *et al.*, 2010). Similarly, LM5, which binds to linear galactan, also exhibited a high avidity to R2U2G2. Although the presence of contaminants in R2U2G2 cannot be totally precluded it cannot explain the high level of recognition of this oligosaccharide by LM5 and LM6. It is more likely that both antibodies cross-react with this peculiar motif. More work is needed to understand this unexpected binding and to evaluate

how it could impact on the interpretation of data describing LM5 and LM6 epitope distribution *in planta*.

RUP antibodies epitope characterization was far from being straightforward. As mentioned above, the 22 RUP hybridomas all produced mAbs that have the same stringent recognition pattern to glycans on micro-arrays. All the main plant cell walls polysaccharides were present on the array but RUP antibodies recognized potato and soybean RGIs only. Tenths of homogeneous, sequence-defined plant oligosaccharide structures were also printed but RUP antibodies did not recognize any of them. Obviously, RUP antibodies epitope was RGI-related but its specific structure was not represented in the oligosaccharides library used. The primary reason for this is the lack of facile methods for the production of sets of homogeneous, sequence-defined plant oligosaccharide structures (Pedersen *et al.*, 2012). Even though much effort has been devoted to the production of a comprehensive set of defined oligosaccharide structures, either by isolation from polysaccharides or by *de novo* chemical synthesis, covering the enormous complexity of cell wall glycans is illusory.

Nevertheless, the implementation of a variant to the epitope detection chromatography technique developed by Cornuault *et al*., (2014) and the linkage analysis of the anion-exchange chromatography-resolved fractions allowed to evidence that the RUP antibodies are likely to be highly specific to oligosaccharides made of (1→4)-linked Gal*p* residues interspersed with internal (1→5)-linked Ara*f* residues. The insertion of (1→5)-α-L-Ara*f* residues in a (1→4)-β-D-Gal*p* backbone had also been detected in soybean RGI (Huisman *et al.*, 2001), which makes sense with the glycan micro-array results.

IV.3. MAb binding *in planta*

IV.3.a Pectin probed by mAbs

MAbs are powerful tools for describing the distribution pattern of cell wall polymers *in planta*. Today several mAbs binding to pectin are available (Table.I.1) and have been used with success to reveal the specific distribution of pectin structural domains at tissue and cell

wall level in transverse sections of various plant material (Willats *et al.*, 2001a) or in a given tissue at different stages of organ development. However, epitope detection depends not only on its occurrence *in planta* but also on its preservation during sample preparation for microscopy and on it accessibility. In potato tuber, removal of galactan was required to be able to detect RUP2 epitope and labeling was further increased by arabinan degradation (Figure.III.8J-K and 9.A-B). The INRA-RU1 epitope restricted to middle lamella in absence of pre-treatment was found throughout the cell wall after galactan digestion (Figure.III.8I). Epitope masking has been described in literature: in tobacco endosperm, INRA-RU1 was revealed using an endo-β-mannanase to remove the abundant heteromannan (Lee *et al.*, 2013). In transverse sections from the second internode of stems of *M.x giganteus*, *M. sacchariflorus* and *M. sinensis*, a xylanase treatment has been shown to promote LM5 binding (Xue *et al.*, 2013). This strongly suggests that preliminary information on the cell wall composition of the material investigated is useful to set up the adequate protocol for unmasking targeted epitopes. In this respect, the semi-quantitative glycan-array methods (Moller *et al.*, 2008) applied to isolated tissues or sub organ level could be helpful for a rapid analysis and complementary to the *in situ* approach. Immunochemistry is a very sensitive method and revealed structure that can be hardly detected by other *in situ* methods such as FT-IR or Raman microspectroscopies. Furthermore, the immunolabeling approach has the advantage of being a high spatial image resolution method but its molecular resolution it is limited by the availability of antibodies, which is confined to a small number of well characterized wall epitopes. Chemical imaging, which combines spatial information with chemical information such as Maldi mass spectrometry imaging could very useful in the future.

IV.3.b Insights on the pectic organization in the wall

Pectins are variable molecules. Motifs recognized by the different mAbs are not uniformly distributed within the cell wall. A schematic view of the distribution of pectin domains within the cell wall can be drawn from the data of available literature completed by some of our results (Figure.IV.1). Methyl esterified HG are present throughout the cell wall (Leboeuf *et al.*, 2005). Arabinan and galactan are found in the primary cell wall but are absent in cell

junctions. Within the primary wall, they do not always colocalize. For example, in sugar beet root (Guillemin *et al.*, 2005), tomato pericarp (Jones *et al.*, 1997; Guillon *et al.*, 2008) and potato tuber (Bush and McCann, 1999; Bush *et al.*, 2001), (1→4)-galactan is localized specifically in the zone close to the plasmalemma whereas arabinan is detected throughout the wall. Close to plasmalemma is also reported RGII in radish and rice roots, cultured tobacco cells, red clover root nodules, and lily growing pollen tubes (Matoh *et al.*, 1998). Unesterified RGI backbone is detected in the middle lamella of tobacco seed endosperm (Lee *et al.*, 2013). In the present study, RGI backbone was found both in middle lamella and in primary cell wall after digestion of neutral side chains. Esterified and low or non-esterified HGs prone to react with Ca^{2+} are generally found confined to middle lamellae, lining intercellular spaces and at cell junction (Willats *et al.*, 2001a). Surprisingly, the RUP2 epitope contrary to (1→5)-arabinan and (1→4)-galactan (Bush *et al.*, 2001; Oomen *et al.*, 2002) is restricted to middle lamella (chapter III). It is interesting to notice that RGI side chains and backbone are not always present in the same region of the wall. Of course, as it was commented before, a non-detected motif may be absent or, just masked or can display a conformation which doesn't allow the mAb recognition. This view of the cell wall is very simplistic as it does not integrate changes in the structural features of pectin with cell development. Nevertheless, these observations raise questions about the linkages between the different pectin domains and the mechanisms by which they are integrated into the wall.

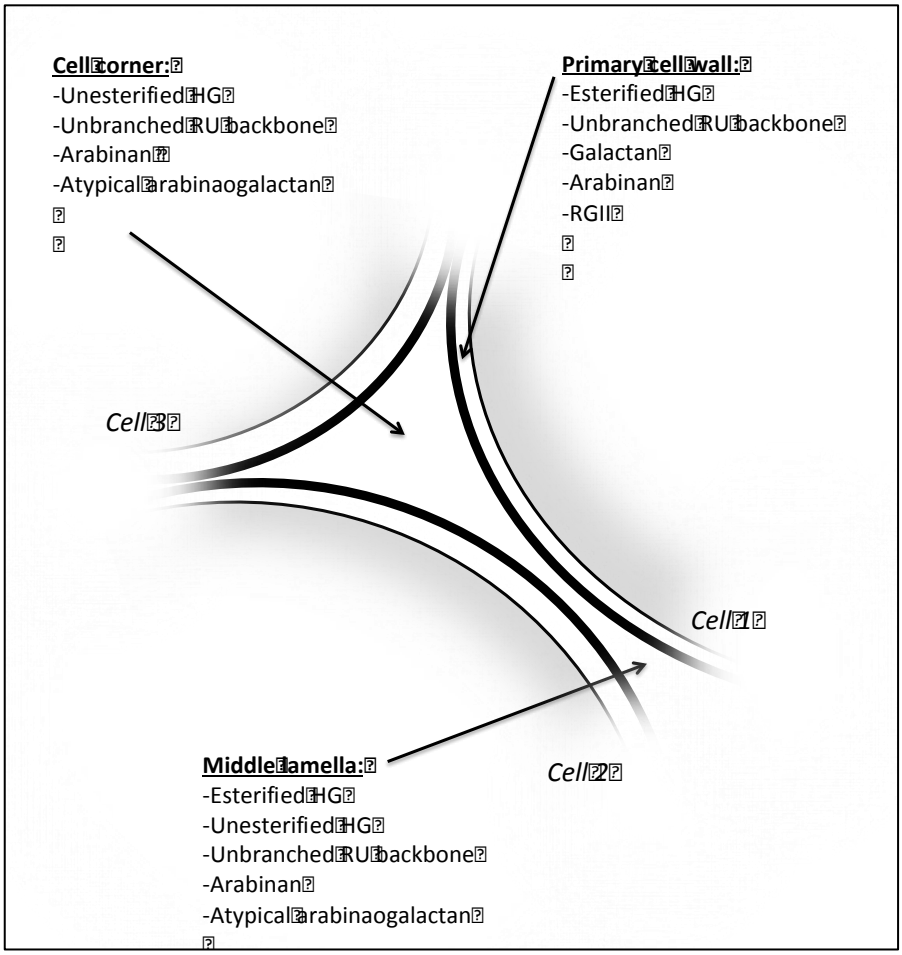


Figure IV.1: Detection of pectic domain in cell walls

References

Bonnin E, Brunel M, Gouy Y, Lesage-Meessen L, Asther M, Thibault JF. 2001. Aspergillus niger I-1472 and Pycnoporus cinnabarinus MUCL39533, selected for the biotransformation of ferulic acid to vanillin, are also able to produce cell wall polysaccharide-degrading enzymes and feruloyl esterases. *Enzyme and Microbial Technology* **28**(1): 70-80.

Bonnin E, Garnier C, Ralet MC. 2014. Pectin-modifying enzymes and pectin-derived materials: applications and impacts. *Applied Microbiology and Biotechnology* **98**(2): 519-532.

Bush MS, Marry M, Huxham IM, Jarvis MC, McCann MC. 2001. Developmental regulation of pectic epitopes during potato tuberisation. *Planta* **213**(6): 869-880.

Bush MS, McCann MC. 1999. Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiologia Plantarum* **107**(2): 201-213.

Clausen MH, Ralet M-C, Willats WGT, McCartney L, Marcus SE, Thibault J-F, Knox JP. 2004. A monoclonal antibody to feruloylated-(1→4)-ß-D-galactan. *Planta* **219**(6): 1036-1041.

Cornuault V, Buffetto F, Manfield I, Ralet MC, Knox JP. 2014. Molecular and biochemical tools for the analysis of glycans in plant cell walls. WallTraC Symposium, Paris, France.

Doco T, Brillouet J-M. 1993. Isolation and characterisation of a rhamnogalacturonan II from red wine. *Carbohydrate Research* **243**(2): 333-343.

Ducasse MA, Williams P, Canal-Llauberes RM, Mazerolles G, Cheynier V, Doco T. 2011. Effect of macerating enzymes on the oligosaccharide profiles of Merlot red wines. *J Agric Food Chem* **59**(12): 6558-6567.

Gao X, Zhi Y, Sun L, Peng X, Zhang T, Xue H, Tai G, Zhou Y. 2013. The inhibitory effects of a rhamnogalacturonan I (RG-I) domain from ginseng pectin on galectin-3 and its structure-activity relationship. *Journal of Biological Chemistry* **288**(47): 33953-33965.

Gro Rydahl M. *Unpublish results*.

Guillemin F, Guillon F, Bonnin E, Devaux MF, Chevalier T, Knox JP, Liners F, Thibault JF. 2005. Distribution of pectic epitopes in cell walls of the sugar beet root. *Planta* **222**(2): 355-371.

Guillon F, Philippe S, Bouchet B, Devaux M-F, Frasse P, Jones B, Bouzayen M, Lahaye M. 2008. Down-regulation of an Auxin Response Factor in the tomato induces modification of fine pectin structure and tissue architecture. *Journal of Experimental Botany* **59**(2): 273-288.

Gur'janov OP, Gorshkova TA, Kabel M, Schols HA, van Dam JEG. 2007. MALDI-TOF MS evidence for the linking of flax bast fibre galactan to rhamnogalacturonan backbone. *Carbohydrate Polymers* **67**(1): 86-96.

Huisman MM, Brul LP, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AG. 2001. The occurrence of internal (1 --> 5)-linked arabinofuranose and arabinopyranose residues in arabinogalactan side chains from soybean pectic substances. *Carbohydrate Research* **330**(1): 103-114.

Jones L, Seymour GB, Knox JP. 1997. Localization of Pectic Galactan in Tomato Cell Walls Using a Monoclonal Antibody Specific to (1-->4)-[beta]-D-Galactan. *Plant physiology* **113**(4): 1405-1412.

Khodaei N, Karboune S. 2013. Extraction and structural characterisation of rhamnogalacturonan I-type pectic polysaccharides from potato cell wall. *Food Chemistry* **139**(1-4): 617-623.

Lau JM, McNeil M, Darvill AG, Albersheim P. 1987. Treatment of rhamnogalacturonan I with lithium in ethylenediamine. *Carbohydrate Research* **168**(2): 245-274.

Leboeuf E, Guillon F, Thoiron S, Lahaye M. 2005. Biochemical and immunohistochemical analysis of pectic polysaccharides in the cell walls of Arabidopsis mutant QUASIMODO 1 suspension-cultured cells: implications for cell adhesion. *Journal of Experimental Botany* **56**(422): 3171-3182.

Leclere L, Cutsem PV, Michiels C. 2013. Anti-cancer activities of pH- or heat-modified pectin. *Frontiers in Pharmacology* **4**: 128.

Lee KJ, Cornuault V, Manfield IW, Ralet MC, Knox JP. 2013. Multi-scale spatial heterogeneity of pectic rhamnogalacturonan I (RG-I) structural features in tobacco seed endosperm cell walls. *Plant J* **75**(6): 1018-1027.

Levigne SV, Ralet MC, Quemener BC, Pollet BN, Lapierre C, Thibault JF. 2004. Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiology* **134**(3): 1173-1180.

Liwanag AJ, Ebert B, Verhertbruggen Y, Rennie EA, Rautengarten C, Oikawa A, Andersen MC, Clausen MH, Scheller HV. 2012. Pectin biosynthesis: GALS1 in Arabidopsis thaliana is a beta-1,4-galactan beta-1,4-galactosyltransferase. *Plant Cell* **24**(12): 5024-5036.

Macquet A, Ralet MC, Kronenberger J, Marion-Poll A, North HM. 2007. In situ, chemical and macromolecular study of the composition of Arabidopsis thaliana seed coat mucilage. *Plant Cell Physiol* **48**(7): 984-999.

Matoh T, Takasaki M, Takabe K, Kobayashi M. 1998. Immunocytochemistry of Rhamnogalacturonan II in Cell Walls of Higher Plants. *Plant and cell physiology* **5**: 9.

Matsunaga T, Ishii T, Matsumoto S, Higuchi M, Darvill A, Albersheim P, O'Neill MA. 2004. Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes, and bryophytes. Implications for the evolution of vascular plants. *Plant physiology* **134**(1): 339-351.

Moller I, Marcus SE, Haeger A, Verhertbruggen Y, Verhoef R, Schols H, Ulvskov P, Mikkelsen JD, Knox JP, Willats W. 2008. High-throughput screening of monoclonal antibodies against plant cell wall glycans by hierarchical clustering of their carbohydrate microarray binding profiles. *Glycoconjugate Journal* **25**(1): 37-48.

Nakamura A, Furuta H, Maeda H, Takao T, Nagamatsu Y. 2002. Structural studies by stepwise enzymatic degradation of the main backbone of soybean soluble polysaccharides consisting of galacturonan and rhamnogalacturonan. *Bioscience, Biotechnology, and Biochemistry* **66**(6): 1301-1313.

O'Neill MA, Eberhard S, Albersheim P, Darvill AG. 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* **294**(5543): 846-849.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annual Review of Plant Biology* **55**: 109-139.

ØBro J, Harholt J, Scheller HV, Orfila C. 2004. Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures. *Phytochemistry* **65**(10): 1429-1438.

Oomen RJFJ, Doeswijk-Voragen CHL, Bush MS, Vincken J-P, Borkhardt B, Van Den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF. 2002. In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. *The Plant Journal* **30**(4): 403-413.

Pabst M, Fischl RM, Brecker L, Morelle W, Fauland A, Kofeler H, Altmann F, Leonard R. 2013. Rhamnogalacturonan II structure shows variation in the side chains monosaccharide composition and methylation status within and across different plant species. *Plant J*: 61-72.

Pedersen HL, Fangel JU, McCleary B, Ruzanski C, Rydahl MG, Ralet MC, Farkas V, von Schantz L, Marcus SE, Andersen MC, Field R, Ohlin M, Knox JP, Clausen MH, Willats WG. 2012. Versatile high resolution oligosaccharide microarrays for plant glycobiology and cell wall research. *Journal of Biological Chemistry* **287**(47): 39429-39438.

Pellerin P, Doco T, Vidal S, Williams P, Brillouet JM, O'Neill MA. 1996. Structural characterization of red wine rhamnogalacturonan II. *Carbohydr Res* **290**(2): 183-197.

Popper ZA, Fry SC. 2008. Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall. *Planta* **227**(4): 781-794.

Ralet MC, Lerouge P, Quemener B. 2009. Mass spectrometry for pectin structure analysis. *Carbohydrate Research* **344**(14): 1798-1807.

Ralet MC, Tranquet O, Poulain D, Moise A, Guillon F. 2010. Monoclonal antibodies to rhamnogalacturonan I backbone. *Planta* **231**(6): 1373-1383.

Renard C, Thibault JF. 1993. Structure and properties of apple and sugar-beet pectins extracted by chelating-agents. *Carbohydrate Research* **244**(1): 99-114.

Reuhs BL, Glenn J, Stephens SB, Kim JS, Christie DB, Glushka JG, Zablackis E, Albersheim P, Darvill AG, O'Neill MA. 2004. l-Galactose replaces l-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the l-fucose-deficient mur1 Arabidopsis mutant. *Planta* **219**(1): 147-157.

Schols HA. 1995. *Structural characterization of pectic hairy regions isolated from apple cell walls*, Wageningen Universiteit.

Schols HA, Posthumus MA, Voragen AGJ. 1990. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research* **206**(1): 117-129.

Séveno M, Voxeur A, Rihouey C, Wu A-M, Ishii T, Chevalier C, Ralet M, Driouich A, Marchant A, Lerouge P. 2009. Structural characterisation of the pectic polysaccharide rhamnogalacturonan II using an acidic fingerprinting methodology. *Planta* **230**(5): 947-957.

Shin K-S, Kiyohara H, Matsumoto T, Yamada H. 1998. Rhamnogalacturonan II dimers cross-linked by borate diesters from the leaves of Panax ginseng C.A. Meyer are responsible for expression of their IL-6 production enhancing activities. *Carbohydrate Research* **307**(1-2): 97-106.

Sørensen I, Willats WG. 2011. Screening and characterization of plant cell walls using carbohydrate microarrays. *Methods Mol Biol* **715**: 115-121.

Spellman MW, McNeil M, Darvill AG, Albersheim P, Dell A. 1983. Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. *Carbohydrate Research* **122**(1): 131-153.

Stevenson TT, Darvill AG, Albersheim P. 1988. Structural features of the plant cell-wall polysaccharide rhamnogalacturonan-II. *Carbohydrate Research* **182**(2): 207-226.

Tan L, Eberhard S, Pattathil S, Warder C, Glushka J, Yuan CH, Hao ZY, Zhu X, Avci U, Miller JS, Baldwin D, Pham C, Orlando R, Darvill A, Hahn MG, Kieliszewski MJ, Mohnen D. 2013. An Arabidopsis Cell Wall Proteoglycan Consists of Pectin and Arabinoxylan Covalently Linked to an Arabinogalactan Protein. *Plant Cell* **25**(1): 270-287.

Thomas JR, Darvill AG, Albersheim P. 1989. Isolation and structural characterization of the pectic polysaccharide rhamnogalacturonan II from walls of suspension-cultured rice cells. *Carbohydrate Research* **185**(2): 261-277.

Verhertbruggen Y, Marcus SE, Haeger A, Ordaz-Ortiz JJ, Knox JP. 2009a. An extended set of monoclonal antibodies to pectic homogalacturonan. *Carbohydrate Research* **344**(14): 1858-1862.

Verhertbruggen Y, Marcus SE, Haeger A, Verhoef R, Schols HA, McCleary BV, McKee L, Gilbert HJ, Knox JP. 2009b. Developmental complexity of arabinan polysaccharides and their processing in plant cell walls. *Plant Journal* **59**(3): 413-425.

Vidal S, Doco T, Williams P, Pellerin P, York WS, O'Neill MA, Glushka J, Darvill AG, Albersheim P. 2000. Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain. *Carbohydr Res* **326**(4): 277-294.

Vidal S, Salmon J-M, Williams P, Pellerin P. 1999. Penicillium daleae, a soil fungus able to degrade rhamnogalacturonan II, a complex pectic polysaccharide. *Enzyme and Microbial Technology* **24**(5-6): 283-290.

Vidal S, Williams P, Doco T, Moutounet M, Pellerin P. 2003. The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers* **54**(4): 439-447.

Voxeur A, Gilbert L, Rihouey C, Driouich A, Rothan C, Baldet P, Lerouge P. 2011. Silencing of the GDP-D-mannose 3,5-epimerase affects the structure and cross-linking of the pectic polysaccharide rhamnogalacturonan II and plant growth in tomato. *J Biol Chem* **286**(10): 8014-8020.

Whitcombe AJ, O'Neill MA, Steffan W, Albersheim P, Darvill AG. 1995. Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II. *Carbohydr Res* **271**(1): 15-29.

Willats WGT, Knox JP, Mikkelsen JD. 2006. Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology* **17**(3): 97-104.

Willats WGT, Marcus SE, Knox JP. 1998. Generation of a monoclonal antibody specific to (1-->5)-α-L-arabinan. *Carbohydrate Research* **308**(1-2): 149-152.

Willats WGT, McCartney L, Mackie W, Knox JP. 2001a. Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology* **47**(1): 9-27.

Willats WGT, McCartney L, Steele-King C, Marcus S, Mort A, Huisman M, Alebeek G-J, Schols H, Voragen AJ, Goff A, Bonnin E, Thibault J-F, Knox JP. 2004. A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta* **218**(4): 673-681.

Xue J, Bosch M, Knox JP. 2013. Heterogeneity and Glycan Masking of Cell Wall Microstructures in the Stems of Miscanthus x giganteus, and Its Parents M. sinensis and M. sacchariflorus. *PLoS ONE* **8**(11).

Zaidel DNA, Meyer AS. 2012. Biocatalytic cross-linking of pectic polysaccharides for designed food functionality: Structures, mechanisms, and reactions. *Biocatalysis and Agricultural Biotechnology* **1**(3): 207-219.

Zheng Y, Mort A. 2008. Isolation and structural characterization of a novel oligosaccharide from the rhamnogalacturonan of Gossypium hirsutum L. *Carbohydrate Research* **343**(6): 1041-1049.

Zykwinska A, Rondeau-Mouro C, Garnier C, Thibault JF, Ralet MC. 2006. Alkaline extractability of pectic arabinan and galactan and their mobility in sugar beet and potato cell walls. *Carbohydrate Polymers* **65**(4): 510-520.

l'université nantes angers le mans
PÔLE DE RECHERCHE ET D'ENSEIGNEMENT SUPÉRIEUR

UNIVERSITÉ DE NANTES

Thèse de Doctorat

Fanny Buffetto

Generation of new monoclonal antibodies to rhamnogalacturonan fragments

Résumé

Les pectines sont largement présentes dans la lamelle moyenne et la paroi primaire des dicotylédones. Elles sont composées de différents domaines structuraux liés de façon covalente. Les rhamnogalacturonanes II et les rhamnogalacturonanes I sont les domaines pectiques les plus complexes. Le rhamnogalacturonane II est formé d'une chaîne principale d'homogalacturonane sur laquelle sont branchées 5 chaînes latérales contenant de nombreux oses rares. Le rhamnogalacturonane I possède un squelette hétérogène de rhamnoses et d'acides galacturoniques. Ce domaine contient des ramifications constituées d'arabinoses et de galactoses. Pour localiser ces structures *in planta*, la microscopie en immunofluorescence est une technique sensible. Les immunomarquages sont réalisés par le biais d'anticorps reconnaissant des structures très spécifiques. Certains anticorps spécifiques des pectines sont déjà disponibles, cependant la production de nouvelles sondes est nécessaire. Pour générer de nouveaux anticorps, des oligosaccharides issus de rhamnogalacturonane II et rhamnogalacturonane I ont été isolés. La préparation de ces oligosaccharides a révélé une grande diversité de motifs structuraux composant ces domaines. Les immunisations menées avec les différents oligosaccharides ont permis la génération d'un nouvel anticorps contre les chaînes latérales des rhamnogalacturonanes I. Cet anticorps reconnaît un motif structural, jusqu'alors très peu décrit dans la littérature, mais présent dans les parois cellulaires riches en galactanes. Cette structure *in planta* a été localisée dans le tubercule de pomme de terre ainsi que dans les jeunes racines d'Arabidopsis. La fonction de cette structure reste encore à déterminer.

Mots clés

Pectine ; rhamnogalacturonane ; anticorps monoclonaux

Abstract

Pectins are widely present in middle lamellae and primary cell walls of dicots. They are composed of different covalently linked structural domains. Rhamnogalacturonan II and rhamnogalacturonan I are the most complex pectic domains. Rhamnogalacturonan II is composed of a homogalacturonan backbone, which carries five different side chains containing rare sugars. Rhamnogalacturonan I has a heterobackbone of rhamnoses and galacturonic acids. This domain contains linear or branched arabinose- and galactose-rich chains. To localize these structures *in planta*, immunofluorescence microscopy is a sensitive technique. Immunolabelling is performed with antibodies recognizing specific structures. Some antibodies, which label pectins are already available. However, the production of new probes is required. To generate new antibodies, oligosaccharides from rhamnogalacturonan I and rhamnogalacturonan II were purified. Structural analyses revealed the high diversity of motifs present in rhamnogalacturoan I. Immunizations carried out with the different oligosaccharides have enabled the generation of a new monoclonal antibody binding to rhamnogalacturonan I side chains. This antibody recognizes a molecular motif present in galactan rich sources which has been poorly described in the literature. This structure was localized *in planta*, in potato tuber and in roots of seedling Arabidopsis. However, function of this structure remains to be determined.

Key Words

Pectin ; rhamnogalacturonan ; monoclonal antibodies