Pectin biosynthesis - identification of glycosyltransferases
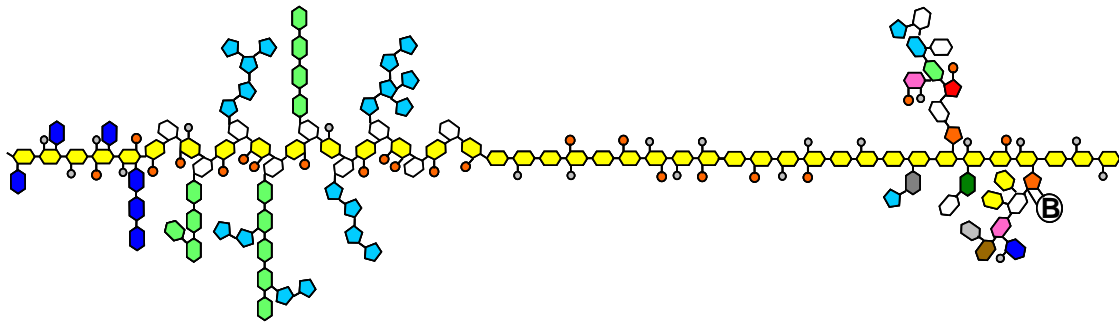
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Content

List of publications

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 Physiol. 140(1):49-58

Orfila C, Sorensen SO, **Harholt J**, Geshi N, Crombie H, Truong HN, Reid JS, 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):613-22

Singh SK, Eland C, **Harholt J**, Scheller HV, Marchant A (2005) Cell adhesion in Arabidopsis thaliana is mediated by ECTOPICALLY PARTING CELLS 1--a glycosyltransferase (GT64) related to the animal exostosins. Plant J. 43(3):384-97

Manfield IW, Orfila C, McCartney L, **Harholt J**, Bernal AJ, Scheller HV, Gilmartin PM, Mikkelsen JD, Paul Knox J, Willats WG (2004) Novel cell wall architecture of isoxaben-habituated Arabidopsis suspension-cultured cells: global transcript profiling and cellular analysis. Plant J. 40(2):260-75

ØBro J, **Harholt J**, Scheller HV, Orfila C (2004) Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures. Phytochemistry 65(10):1429-38

Preface

This thesis is part of the requirement for obtaining the degree: Doctor of Philosophy (PhD). This thesis summarises my work carried out between December 2002 and June 2006 at the Department of Plant Biology, Section for Plant Biochemistry at the Royal Veterinary and Agricultural University, Denmark. My supervisor in the course of my study has been Professor Henrik Vibe Scheller.The purpose of this project was to identify and characterise glycosyltransferases involved in the plant cell wall biosynthesis, with particular focus on pectin biosynthesis.

The first two chapters consist of an introductory review on pectin structure and function *in muro* and a review on our current understanding of the plant cell wall biosynthesis. Chapter 3-7 consist of manuscript published during my study and chapter 8 and 9 are manuscripts in preparation.

Jesper Harholt

Abstract

Glycosyltransferase (GT) Family 47 in the CAZy database was chosen as target genes for a reverse genetic approach to identify GT's involved in plant cell wall biosynthesis. That GT family 47 members were used was based on the presence of a large number of plant genes and already identified cell wall related GT's. The *Arabidopsis* used for the analysis were T-DNA mutants available through either the SALK Institute or Syngenta. After identification of homozygote T-DNA mutants, a cell wall analysis screen based on alcohol insoluble residue from leaves or stem, was performed. For this screen a new method was developed for the sugar composition analysis (Chapter 3). Using this approach two mutants were identified with altered cell wall sugar composition.

A T-DNA mutant of At2g35100 placed in subgroup B, showed a 30 % and 50 % reduction in leaf and stem arabinose content, respectively. Further biochemical characterization of the cell wall using cell wall fractionation, linkage analysis and immunololabelling showed that the reduced arabinose content was due to a reduction in arabinan. This led to a proposed function of At2g35100 as an arabinan arabinosyl transferase (Chapter 7).

A T-DNA mutant of At5g33290, a member of subgroup C, was identified as having reduced xylose content in leaves. After fractionation and purification of cell wall components it was shown that the reduction in xylose content was due to a reduced xylose content in RG-I. An altered rhamnose to galacturonic acid ratio in the endopolygalacturonase treated RG-I was also observed. Linkage analysis of RG-I showed that all xylose residues were terminal. No xylogalacturonan could be detected using xylogalacturonan endohydrolase (see also below). At5g33290 was suggested to be a xylogalacturonan xylosyl transferase based on the biochemical phenotype of the mutant (Chapter 9).

Xylogalacturonan had not previously been described in Arabidopsis leaves. A detailed analysis of xylogalacturonan was performed in collaboration with Joris Zandleven and Gerrit Beldman from Wageningen University. Pectin was extracted from cell wall material isolated from leaves of Arabidopsis and subsequently characterized using xylogalacturonan endohydrolase. MS analyses of the obtained oligomers showed that

xylogalacturonan was generally present in the cell wall in lowly substituted form (Chapter 8).

This thesis also covers the characterization of *ectopically parting cell 1* (*epc1*) a member of the CAZy GT family 64 (Chapter 5). T-DNA mutation of *EPC1*, results in plants with a dramatically reduced growth habit, defects in vascular formation and reduced cell–cell adhesion in hypocotyl and cotyledon tissues. Cell wall composition analysis showed an increased glucose content, properly originating from callose deposits which could be detected in hypocotyl and cotyledon tissues.

Biochemical characterization of *quasimodo 1* (*qua1*) a CAZy GT family 8 member showed a reduction in homogalacturonan and xylan content in the stem. Measurements of the endogenous activity of homogalacturonan synthase and xylan synthase showed in both cases a reduced activity in *qua1* compared to wild type, indicating a co-regulation in the synthesis of homogalacturonan and xylan (Chapter 6).

Cell suspension cultures habituated to isoxaben show reduced cellulose and an increased pectic and hemicellulosic content in the cell wall compared to controls with out isoxaben. Using comparative transcriptomics by DNA-microarrays using habituated and non treated cells novel genes implicated in cell wall assembly were identified. Our understanding of the activity of known cell wall-related genes including glycosyltransferases involved in cellulose and pectin biosynthesis was also extended (Chapter 4).

Dansk resume

Glycosyltransferase (GT) family 47 fra CAZy databasen blev valgt som målgruppe for reverse genetiske eksperimenter, med henblik på at kunne identificere glycolsyltransferaser involveret i syntese af plantecellevæggen. CAZy familie 47 blev valgt på grund af det store antal plante gener til stede i familien, samt at der allerede var identificeret gener involveret i plante cellevægs biosyntesen, i denne familien.

De *Arabidopsis* linier der blev brugt til analyserne var T-DNA mutanter, tilgængelige gennem SALK institute eller Syngenta. Efter identificering af homozygote T-DNA mutanter blev cellevæggen fra blade og stængler analyseret. En ny metode til bestemmelse af kompositionen af alkohol uopløselige dele (Kapitel 3). Ved brug af denne analysemetode blev der identificeret to mutanter med ændret cellevægs sammensætning.

T-DNA mutanten af At2g35100, placeret i undergruppe B i familie 47, havde en 30 % og 50 % reduktion af arabinose indholdet i henholdsvis blade og stængler. Yderligere biokemisk karakterisering af mutanten vha. cellevægsfraktionering, bindingstype analyse og immunofarvninger viste at faldet i arabinose indhold skyldtes et reduceret arabinan indhold. Dette ledte frem til en mulig funktion af At2g35100 som en arabinan arabinosyl transferase (Kapitel 7).

En T-DNA mutant i undergruppe C i familie 47, At5g33290, blev identificeret som en xylose reduceret mutant. Efter fraktionering og oprensning af forskellige cellevægs komponenter blev det vist at det reducerede xylose indhold skyldtes et fald i indhold af xylogalacturonan. En ændret galacturonsyre til rhamnose ratio i RG-I efter endopolygalacturonase behandling blev også observeret. Bindingstype analyse af RG-I viste at faldet i xylose indhold skyldtes et fald i terminal xylose. I mutanten kunne der ikke detekteres noget xylogalacturonan hvis pektin fra mutantens cellevæg blev forsøgt fordøjet med xylogalacturonan endohydrolase (se også nedenfor). Baseret på den biokemiske karakterisering blev aktiviteten af afledte protein fra At5g33290 foreslået at være en xylogalacturonan xylosyltransferase (Kapitel 9).

Xylogalacturonan er ikke tidligere beskrevet i *Arabidopsis* blade. En detaljeret analyse af xylogalacturonan blev foretaget i samarbejde med Joris Zandleven og Gerrit Beldman fra Wageningen Universitet. Pektin fra *Arabidopsis* blade blev oprenset og efterfølgende

karakteriseret vha. xylogalacturonan endohydrolase. MS analyse af de opnåede oligomerer viste at xylogalacturonan var almindeligt tilstede i en lavt substitueret form (Kapitel 8).

Denne afhandling dækker også karakteriseringen af *ectopically parting cell 1* (*epc1*) et medlem af CAZy glycosyltransferase familie 64. T-DNA mutation af *EPC1* medfører en kraftigt reduceret vækst, mangler i dannelsen af det vaskulære væv og reduceret celle til celle adhæsion. Cellevægs kompositions analyse viste et forøget glukose indhold, muligvis fra øget afsætning af callose i cellevæggen (Kapitel 5).

Biokemisk karakterisering af *quasimodo 1* (qua1) viste en reduktion i homogalacturonan og xylan indholdet i stængelen. Målinger af den endogene aktivitet af homogalacturonan syntase samt xylan syntase, viste at disse aktiviteter var reduceret i *qua1* sammenlignet med vild type. Dette kunne indikere en co-regulering af biosyntesen af homogalacturonan og xylan (Kapitel 6).

Isoxaben inhiberer cellulose biosyntesen. Cellesuspensionskulturer behandlet med isoxaben reagerer ved at øge biosyntesen af pektin og hemicelluloser. Ved at sammenligne transkriptomet, vha. DNA-microarray, af de isoxaben behandlede og ikke behandlede cellesuspensionskulturer blev gener muligvis involveret i cellevægsbiosyntesen fundet. Vores forståelse af aktiviteten af kendte glycosyltransferaser blev desuden større (Kapitel 4)

Abbreviations

General abbreviations:

EPG	Endo-polygalacturonase
FAE	Ferulic acid esterase
HG	Homogalacturonan
JIM5	Antibody recognizing low methylated HG
JIM7	Antibody recognizing highly methylated HG
LM5	Antibody recognizing β-1,4-galactan
LM6	Antibody recognizing α-1,5-arabinan
MHR	Modified hairy region
PME	Pectin methyl esterase
RG	Rhamnogalacturonan
RGase	Rhamnogalacturonan hydrolase
RG lyase	Rhamnogalacturonan lyase
XGA	Xylogalacturonan

Abbreviations for sugars:

Ara	Arabinose
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
Glc	Glucose
GlcA	Glucuronic acid
Man	Mannose
Rha	Rhamnose
Xyl	Xylose
f	Furanose form of the sugar

Chapter 1

This chapter consist of a review on pectin structure and function *in muro*. The new model for pectin superstructure is discussed.

The Structure of Pectin and its Function *in muro*

Introduction

With only a few exceptions all plant cells are surrounded by a cell wall. The cell wall contributes to the form and properties of the encompassed cell, ensures a mechanical base for upward growth and is the first barrier met by compounds and invading organisms. Two different cell walls have been established, the primary cell wall surrounding all cells and the secondary cell wall which is deposited between the plasmamembrane and the primary cell wall. The secondary cell wall is mainly a structural component, enhancing the mechanical strength of the cell wall. The cell wall itself is a complex structure in which cellulose microfibrils are embedded in a matrix of glycans, phenolic compounds, glycoproteins, proteoglucans, low molecular weight compounds and ions. The embedding glycans in the primary wall consists of mainly pectin and xyloglucan in dicots and non-commelinoid monocots (**Figure 1**).
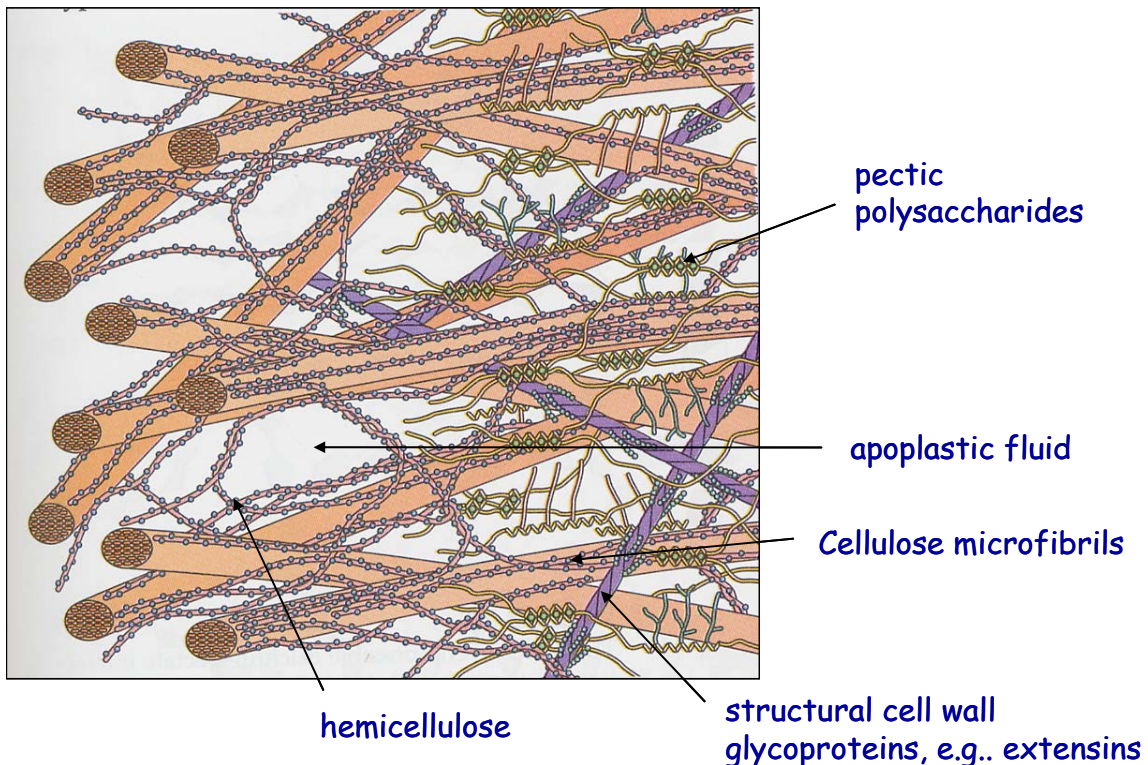
Figure 1 A three-dimensional molecular of a dicot cell wall which shows the molecular interactions between cellulose, xyloglucan and pectin. The framework of cellulose and xyloglucan is embedded in a matrix of pectin. PGA junction zone: Ca^{2+} mediated crosslink of low methylated homogalacturonan. (From Carpita and Gibeaut 1993).

In commelinoid monocots xylan is the main embedding component. Within the monocots, the Poales furthermore have mixed linkage β-glucan as part of the matrix polymers. In the secondary cell wall the main embedding glycan is xylan in both mono- and dicots. In the primary cell wall of dicots the most abundant matrix glycan is pectin. Pectin is furthermore present in the middle lamella more or less outside the primary cell wall, conferring strength and plasticity to the cell to cell adhesion. The following description of the pectin structure covers many features that are specific for certain plant species. Extrapolation and establishment of a general structure model from a single species applicable to whole plant genera is not possible. Certain structural motifs e.g. the ferulyolation of the arabinan side chains, have been found in several species in the *Chenopodiaceae* and it is plausible that the whole family has this structural motif (Ishii 1997b).

The review presented here is focused on the structure and functionality of the pectin molecule in the primary cell wall of dicots. Much can also be written on the structure and functionality of pectin when used outside the plant in industrial applications as e.g. a gelling agent in the food industry or as coating material in the pharmaceutical industry. That is however beyond the scope of this review.

Pectin structure

Galacturonans

The galacturonans in the pectin structure are distinguished by their linear α-1,4 linked GalA backbone. The most abundant of the galacturonans is the homogalacturonan (HG), which, as the name indicate, consist only of a GalA backbone. The GalA moiety of the homogalacturonan can be esterified with a methyl group at the C-6 carboxy group. Acetylation at the C-2 or C-3 has also been reported (Ishii 1997a)(Figure 2).

The HG is hypothesised to be deposited into the cell wall as a highly methylated polymer. After deposition, cell wall localized pectin methyl esterases (PME) are thought to remove methyl groups from the HG (O'Neill *et al.*, 1990).

The action of plant PME's leads to stretches of HG without methylation giving rise to a non-random distribution of the methylation of the HG (Limberg *et al.*, 2000). The acetylation pattern of HG seems to be more random in sugar beet and spinach HG (Ralet *et al.*, 2005 and Perrone *et al.*, 2002). The HG backbone may (Powell *et al.*, 1982) or may not (Zhan *et al.*, 1998) be interrupted by occasional single Rha residues. (See also discussion later in pectin superstructure paragraph).

Substituted galacturonans with sugars linked to the GalA backbone via a glycoside linkage exist in several forms. Xylogalacturonan (XGA) has β-1,3 linked Xyl attached to the GalA backbone (Figure 2). The Xyl can either be a single residue or a chain of β-1,4-linked Xyl residues. The xylosidation of the HG backbone can be quite extensive, with up to 75 % of the GalA residues xylosidated (Visser and Voragen, 1996). XGA was first detected in mountain pine pollen (Bouveng 1965) and has since been found in various seeds, fruits and storage tissues (Matsuura and Hatanaka 1988). Using an antibody (LM8) raised against XGA from pea hull, XGA has also been discovered the rootcap of *Arabidopsis*, with possible involvement in cell detachment (Willats *et al.*, 2004). This hypothesis is supported by the finding of the same XGA epitope in the septum of *Arabidopsis* siliques (Sørensen *et al.*, In preparation, Chapter 9). Until recently XGA had not been reported in vegetative tissue of *Arabidopsis* although terminal Xyl was found in RG-I preparations (Zablackis *et al.*, 1995).

Through the use of XGA endohydrolase, XGA was found in vegetative tissue of *Arabidopsis* (Zandleven *et al.*, In preparation, Chapter 8). The LM8 antibody does not recognize the XGA present in vegetative tissue from *Arabidopsis* indicating that several structural motifs of xylogalactoronan can be found, possibly with varying degree of Xyl substitution (Sørensen *et al.*, In preparation, Chapter 9). Apiogalacturonan is a substituted galacturonan with β-D-Api*f* and β-D-Apif-(1,3)-β-D-Api*f* linked to the C-2 on GalA. Apiogalacturonan has been found in the aquatic monocots *Lemna sp.* and *Zostera sp.* (Duff 1965).

The conformation of HG seems in all its states to be an extended and flexible polymer. It can appear as 2_1 and 3_1, where N is the number of repeating units per turn of the helix and $_1$ is a right-handed and $_2$ is a left-handed helix, which has been shown by measurements of solid state HG (Jarvis and Apperley 1995). By molecular modelling it has also been shown that 3_2 and 4_1 are feasible low energy conformations.

In all four conformations the extended HG has a pitch per residue of approximately 0.435 nm. Methylation and acetylation of the HG do not seem to alter the conformational possibilities (Perez *et al.*, 2000). Insertion of single or double Rha residues, interspaced by a GalA residue, has been proposed to introduce kinks into the HG chain. If a third Rha is introduced the kink is reduced and the structure resembles RG-I (Engelsen *et al.*, 1996).

Rhamnogalacturonan I

Rhamnogalacturonan I (RG-I) is characterized by its backbone of the repeating disaccharide -4)-α-D-GalA-1,2-α-D-Rha-(1-. The Rha residues can carry side chains of neutral and acidic sugars positioned at the C-4 (Figure 2). The degree of substitution of RG-I varies depending on plant species, tissue type and extraction method. Typically between 20 - 80 % of the Rha residues are substituted at the C-4 (O'Neill *et al.*, 1990). The GalA residues can be acetylated at the C-2 and/or at the C-3 (Ishii 1997a). There is no clear evidence for methylation of the GalA residues in the RG-I backbone as no pure RG-I with methylated GalA residues has been isolated, though RG-I enriched cell wall fraction from flax contained methyl-ester (Rihouey *et al.*, 1995). The GalA in RG-I can furthermore have β-D-GlcA attached to the C-3 (Renard *et al.*, 1999).

Modelling of the structure of RG-I indicate that it is present in a 3_1 extended conformation and that the repeating disaccharide motif assures that kinking of the backbone will not occur (Engelsen *et al.*, 1996).

Arabinan and Galactan

The side chains linked to the C-4 of the Rha residues in the RG-I consist of mainly Ara and Gal residues with smaller amounts of Fuc, GlcA and methyl-GlcA (O'Neill *et al.*, 1990, Showalter 2001)(Figure 2). The major component in what is called the hairy region of pectin is arabinan with linear chains of α-1,5-Ara*f* with branches of single α-1,3-Ara*f* or α-1,3-linked chains of α-1,5-Ara*f* and galactan with linear chains of β-1,4-Gal with branches of single β-1,3-Gal or β-1,4-linked chains of β-1,4-Gal. Arabinogalactan I with a linear β-1,4-Gal chain with single α-1,3-Ara*f* residues can also be present as can arabinogalactan II composed of β-1,3-Gal backbone, containing short side chains of β-1,6-Gal with possible additional of α-1,3-Ara*f* linked to the side chains (Ridley *et al.*, 2001, Showalter 2001). The idea of arabinogalactan II being covalently linked the RG-I is based on co-extraction of the two polymers and could be the result of a tight, but non-covalent interaction with RG-I as arabinogalactan II is also the carbohydrate moiety of arabinogalactan proteins. Proteins can be difficult to extract from cell wall preparations and are present even after phenol/acetic acid/water extraction (Zablackis *et al.*, 1995). The variation in arabinan and galactan structure is large and depends on the plant species, tissue type and physiological state of the cell. Arabinan and galactan can furthermore have ferulic acid esters attached (Fry 1982).

Short galatan sidechains are also residing on arabinan (Øbro *et al.*, 2004, Chapter 3). Galactan and arabinogalactan I can further be glycosylated with GlcA and Fuc (O'Neill *et al.*, 1990) and arabinogalactan II can have a range of sugars, in smaller quantities, attached to the side chains (Showalter 2001).

Xylogalacturonan   **Rhamnogalacturonan I**   **Homogalacturonan**   **Rhamnogalacturonan II**

⬡ = **D-Galacturonic acid**   ⬠ = **L-Arabinose**   ⬠ = **D–Apiose**   ⬥ = **O-Acetyl**

⬡ = **L-Rhamnose**   ⬠ = **D-Galactose**   ⬠ = **L-Fucose**   ⬦ = **O-Methyl**

⬣ = **D-Glucuronic acid**   ⬟ = **L-Aceric acid**   ⬣ = **D-Xylose**   Ⓑ = **Borate**

⬣ = **Kdo**   ⬣ = **D-Dha**   ⬡ = **L-Galactose**
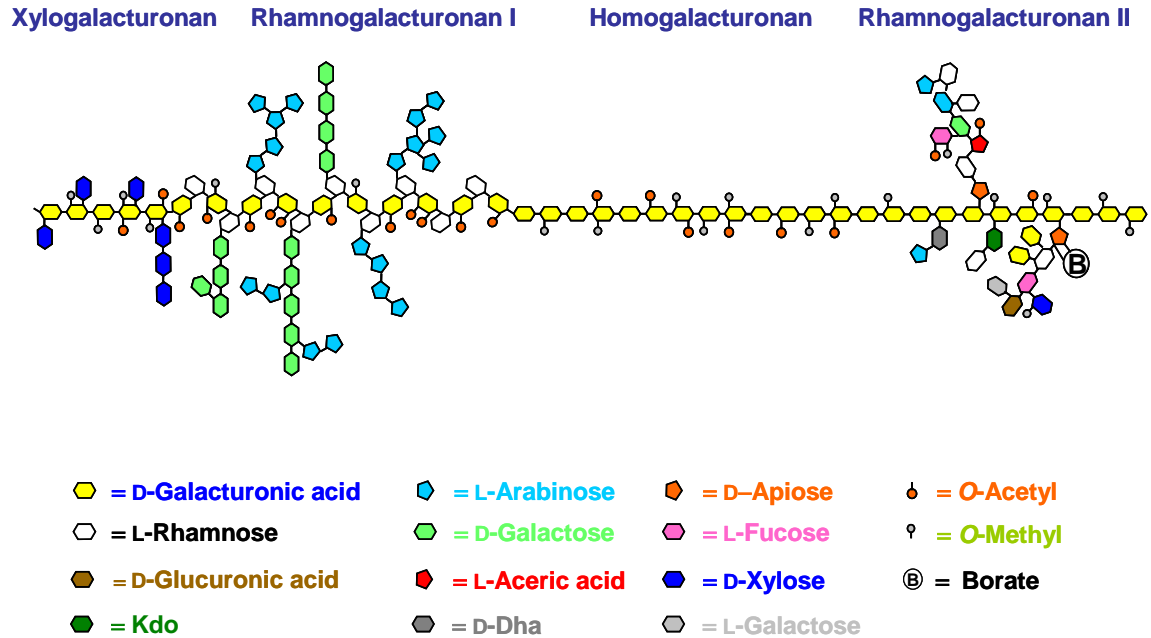
Figure 2 Simplified model of the pectin superstructure.

Arabinan has been modelled to be a 3_1 extended chain attached to the RG-I at a 50° angle. Galactan appears to be a more open helix with a 5_1 structure placed perpendicular to the RG-I backbone. In both cases the extended arabinan and the perpendicular galactan leaves ample space for possible side chains (Engelsen *et al.*, 1996).

Rhamnogalacturonan II

The structure of Rhamnogalacturonan II (RG-II) is highly complex (Figure 3). RG-II is composed of 12 different sugars including the rare 3-deoxy-D-manno-octulosinic acid (Kdo), 3-deoxy-D-lyxo-2-heptulosaric acid (Dha) and Apiose. RG II consists of four chains named A-D residing on a GalA backbone resembling the HG. This backbone is at least eight residues long (Whitcombe *et al.*, 1995). The position of the chains on the GalA backbone has been predicted to be placed with the B-chain, four residues in, counting from the reducing end; the D-Chain five residues in; the A-chain six residues in and lastly the C-Chain placed somewhere between the reducing end and the B-chain (O'Neill *et al.*, 2004).

Figure 3 The glycosyl sequence of RG-II. Naturally occurring variation in the sequence is shortly described along with the changes induced by the *mur1* and *nolac-H18* mutations. (From O'Neill *et al.*, 2004).

Pectin superstructure

Pectin superstructure is a field within the structural characterization of pectin in which there are many unknowns and a large amount of debate. Until 2003, the superstructure of pectin was depicted to be a linear chain of HG interspaced with RG-I backbone. RG-II was suggested to be located on the HG backbone and the hairs on RG-I would then extend more or less perpendicular to the HG/RG-I backbone (Visser and Voragen 1996, Engelsen *et al.*, 1996)(Figure 2). XGA was present in pectin not as a characteristic polymer but more as substituted HG and would be located near the RG-I backbone as XGA and RG-I coeluted after endopolygalacturonase (EPG) treatment of pectin (Thibault *et al.*, 1993). In 2003 another model for the pectin structure was proposed (Vincken *et al.*, 2003).

The most pronounced change in the new model for pectin was that HG and XGA were suggested to be sidechains on the RG-I backbone located along side arabinan and galactan (Figure 4).
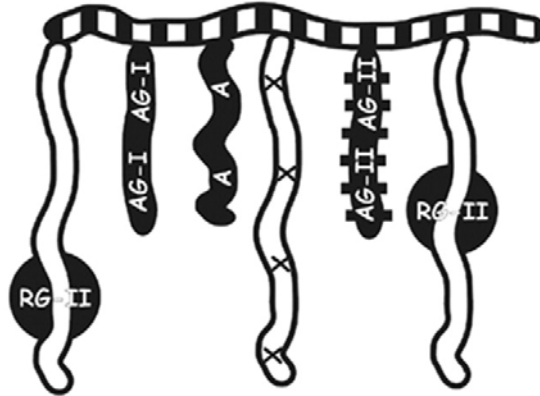
Figure 4 The pectin superstructure as suggested in Vincken *et al.*, 2003. Note the location of HG and XGA as side chains on the RG-I backbone. The exact distribution of the side chains on the RG-I backbone is depicted randomly but could also be distributed in clusters. (From Vincken et al 2003).

This new pectin model was based on analysis of the resulting polymers after hydrolysis of modified hairy regions (MHR) by rhamnogalacturon lyase (RG lyase) or rhamnogalacturonanase (RGase). MHR is a pectin fraction originating from apple treated with a mix of pectin, hemicellulose, and cellulose degrading enzymes. After isolation of MHR from the apple pulp, the MHR was saponified in order to remove acetyl and methyl groups from GalA. The characteristic of MHR are domains consisting of XGA and RG-I with the majority of the galactan removed (Schols *et al.*, 1990b and Schols *et al.*, 1995). After RGase or RG lyase treatment, three distinct pools of polymers could be obtained after size exclusion chromatography. The pool of polymer with the largest molecular mass consisted almost exclusively of XGA and RG-I with arabinan attached. An intermediate sized pool of polymers consisted of RG-I with arabinan sidechains and the smallest polymers were RG-I backbone oligomers with single Gal residues attached and only small amount of arabinan (Schols *et al.*, 1990a, Mutter *et al.*, 1998). The intermediate pool had a GalA to Rha ratio up to four. That the GalA to Rha ratio was not 1 indicated the presence of GalA residues not part of the RG-I backbone. That possible single inserted Rha residues can be present in the HG backbone has been the issue of several publications and some results indicate that it is not the case (Zhan *et al.*, 1998).

Therefore the GalA not residing in the RG-I backbone was proposed to be placed as external oligomers (Vincken *et al.*, 2003). After mild acid hydrolysis thereby removing arabinan, the external galA oligomers were susceptible to EPG treatment (Vincken *et al.*, 2003). This observation leads to the suggestion that GalA oligomers were sidechains of the RG-I as removal of possibly sterical hindrance from arabinan lead to EPG susceptibility. XGA was also proposed to be side chains of RG-I as degradation with either XGA exo- or endohydrolases lead to the same decrease in the molecular size of modified hairy region.

There are results that indicate that the conclusions drawn from the above presented results are somewhat uncertain. These results are both present in the publications used for suggesting the new pectin model and newly acquired data. The main argument for the presence of HG as side chains of RG-I is that a GalA to Rha ratio above one could be observed in RGase or RG lyase treated MHR. The calculated GalA to Rha ratio of four is not taking the occurrence of XGA into account. The substitution degree in XGA from MHR has been proposed to be 70 % and all the Xyl present was terminally linked (Schols *et al.*, 1995). If the Xyl in the intermediate sized pool is included a GalA to Rha ratio is calculated with the GalA located in XGA subtracted, the actual GalA to Rha ratio is below 1.5. If the GalA to Rha ratio is calculated, again taking XGA into account, on saponified MHR before RGase or RG lyase digestion the GalA to Rha ratio is close to 1 (recalculated from Schols et al 1990a, Mutter *et al.*, 1998). The mild acid hydrolysis facilitating the removal of arabinan and the following susceptibility to EPG degradation can also be questioned as the saponification of MHR also lead to increased susceptibility to EPG (Schols et al 1990b). It is not clear from Vincken *et al.*, 2003 whether the saponified MHR was EPG digested before acid hydrolysis of arabinan.

While the effect of exoPG and XGA endohydrolase treatment on XGA led to the proposal that XGA is a side chain of RG-I, it could also be explained by placing XGA solely on the non-reducing side of RG-I. If all XGA were located there, the same shift in molecular size would be expected using both XGA degrading enzymes as exoPG degrade XGA from the non-reducing end. There has not to the authors knowledge been published any data on the localization of XGA relative to RG-I.

If Rha was present in the HG or XGA backbone as single insertions RGase or RG lyase could possibly digest it and thereby produce smaller oligomers that would interfere with the GalA to Rha ratio as described above. During the characterization of XGA in *Arabidopsis* (Zandleven *et al.*, In preparation, Chapter 8) a GalA-GalA-Rha-GalA-GalA-Xyl oligomer was found (Zandleven *et al.*, Unpublished). The exact order of the sugars was determined by MS/MS but the linkages are not known. The oligomer properly originate from XGA as it was found after xylogalacturon endohydrolase treatment of pectin and the sugars present indicate that it is indeed a XGA fragment. This finding cannot be readily extrapolated to conclude that single inserted Rha residues are present in HG also.

Concluding remarks on pectin structure

With the above description of the pectin fine and superstructure The author have tried to describe all the major features of pectin. However, a lot of variation in the structure exists and structures not described in this review also occur. One example could be the structure of arabinan and galactan. The major structures are the β-1,4-Gal*p* and α-1,5-Ara*f* based backbone of galactan and arabinan, respectively. Side chains can then be added to these backbones. The variation over this rather simple theme is enormous and depends on plant species, tissue type and stage of cell development. The only structure in pectin that is highly conserved is RG-II. The variation in RG-II among vascular plants is restricted to methylation of two specific sugars in pteridophytes and lycophytes and the absence of two particular terminal sugars in certain species e.g. *Arabidopsis* (O'Neill *et al.*, 2004). The ongoing debate on the pectin super structure has yet to be resolved. So far substantial circumstantial evidence for both models has been presented. But what is more pressingly needed is structural components that unambiguously point to either of the two models e.g. a Rha with 1, 2, 4 linked GalA or a (GalA-Rha)$_n$-GalA-GalA-GalA fragment. Ongoing work using limited acid hydrolysis of MHR and subsequent structural analysis by HPAEC coupled with MALDI-TOF post source decay or ESI MS^n are aimed at identifying a key structural element (Coenen et al, Unpublished work presented at the Xth Cell wall meeting, 2004).

Localisation

Pectin is proposed to be part of all primary cell walls (Willats *et al.*, 2001). Furthermore pectin can be found outside what is normally considered primary cell wall e.g. in the seed mucilage (Penfield *et al.*, 2001).

However, the abundance of pectin in the wall can vary significantly. Furthermore there are differences in the relative amounts of the different pectic structures, both between cells and within the cell wall. Some structures have only been reported in specific species or cell types. This negative result could however also reflect that the amount is below detection level or that the target polymer is masked by other cell wall components. In order to identify specific polymers, stringent methods of discriminating between structures should be used. Two strong approaches are: immunoflourecent labeling of monoclonal antibodies raised against well-defined oligomers; or use of pure well-characterized hydrolytic enzymes. Examples of both techniques are found in the detection of XGA outside fruit or seed tissue.

We have shown that XGA is present in *Arabidopsis* and is especially abundant in adult leaves, based on digestion of pectic extracts with XGA endohydrolase (Zandleven *et al.*, In preparation, Chapter 8). Cell wall material isolated from adult leaves from *xgd1-1* and wild type was analyzed for the presence of xylogalacturonan using the same procedure. While XGA oligomers could be detected in the extract from wild type tissue, no XGA oligomers were detected in the *xgd1-1* extract. A subsequent analysis of the distribution of xylogalacturonan by use of the xylogalacturonan specific monoclonal antibody LM8, which has previously been shown to label part of the root tip in *Arabidopsis* (Willats *et al.*, 2004), resulted in an interesting observation. A detailed immunofluorescence analysis of the *Arabidopsis* plant showed labelling in the root tip and very distinct labelling in certain cells in the septum between locules in the siliques in both wild type and *xgd1-1* (Figure 5). The LM8 antibody did not give rise to any labelling in adult leaves in the wild type, where the XGA susceptible to XGA endohydrolase degradation is most abundant. The author suggest that LM8, which has been raised against XGA isolated from pea (*Pisum sativum* L.) testae, binds to a highly substituted epitope of XGA, possibly substituted with a disaccharide of Xyl that is not susceptible to cleavage by XGA endohydrolase used in this work (Sørensen *et al.*, In preparation, Chapter 9).
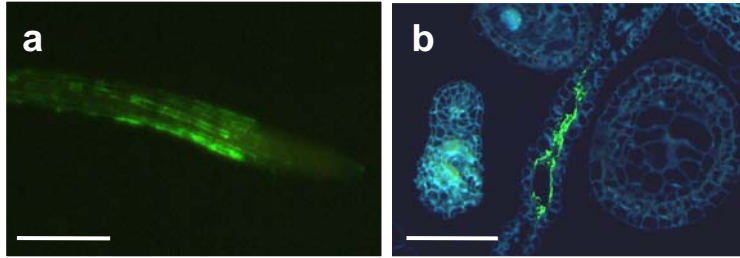
Figure 5 Indirect immunofluorescence labelling of intact roots (a) and transverse sections of resin embedded siliques (b). Roots and siliques were labelled with an antibody with specifictity for xylogalacturonan (LM8). In sliques, the LM8 epitope was restricted to certain cells in the septum between locules as indicted by the arrow. Scale bar =100 μm. (Adapted from Sørensen _et al._, In preparation, Chapter 9)

This study illustrates that different methods can be used and that the results obtained are dependent on the specificity of the method used. The resolution that can be obtained using hydrolytic enzymes are generally lower when compared to immunolabeling methods. This is true even with techniques where single cells are laser dissected out of tissue and oligomers resulting from hydrolytic enzymes are analysed by MS (Markus Pauly, Unpublished results).

As part of all primary cell walls, the HG and RG-I backbone is present in all living plant cells. The nature of HG is not static during the cells life cycle. Highly methylated HG (JIM7 reactive, Willats _et al._, 2000, Clausen _et al._, 2003) is located throughout the cell wall in both developing and matured cell (Bush and McCann 1999, Guillemin et al 2005). Later, during elongation and maturation of the cell wall, low methylated HG (Knox _et al._, 1990, Clausen _et al._, 2003) appears in cell corners and in the walls surrounding inter-cellular spaces, nearest the middle lamella (Bush and McCann 1999, Guillemin _et al._, 2005). Vascular tissue also seems to have an elevated level of low methylated HG (Guillemin _et al._, 2005).

Much is also known about galactan and arabinan due to LM5 and LM6. Galactan appears after the initial cell division in meristems or cambial layers during the cell elongation and differentiation stage (Willats _et al._, 1999, Bush and McCann 1999, McCartney _et al._, 2000 and Ermel _et al._, 2000). The LM5 epitope is reported to be reduced in amount during secondary cell wall formation (Ermel _et al._, 2000), but the reduction in epitope could be due to masking as other results contradict the observed reduction (Guillemin _et_

al., 2005). Galactan is mainly located in an area near the plasma membrane within the wall (Willats *et al.*, 1999, Bush and McCann 1999, McCartney *et al.*, 2000 and Ermel *et al.*, 2000). Arabinan has, as opposed to galactan, been detected in all cells investigated, though there are differences in abundance as e.g. cortical and epidermal cell types have an increased amount of arabinan in the walls compared to parenchymal cells. Meristematic and cambial cells also seem to have an increased content compared to elongating and differentiating cells (Willats *et al.*, 1999, Guillemin *et al.*, 2005, Bush and McCann 1999, McCartney *et al.*, 2000, Bush *et al.*, 2001). Arabinan is detected through out the primary wall and in the middle lamella in areas where contact is made with other cells. Around intracellular spaces and in cell corners there is no staining of the middle lamella (Guillemin *et al.*, 2005, Bush and McCann, 1999).

RG-II is, as with HG and RG-I, considered present in all primary cell walls (O'Neill *et al.*, 2004). RG-II is distributed from the plasma membrane, where it is most abundant, to the middle lamella where it is almost absent (Matoh *et al.*, 1998).

The function of pectin

Intra and intermolecular interactions

Most of the knowledge about pectin properties comes from in vitro studies. Because of the importance of pectin as a food ingredient much is known about isolated pectin in aqueous solutions.

The most studied interaction is the association of HG with other HG's located on the same or another pectin molecule. This particular interaction is also interesting in the application of pectin as a gelling agent, as this is the main interaction involved in forming the advantageous properties of pectin. HG dimerizes by two different types of interactions, depending on the degree of methylation of HG.

Low methylated pectin can gelatinize in vitro by addition of Ca^{2+} to the pectin solution. This gelatinization is achieved due to HG dimerization. The HG dimerizes by hydrogenbonding, van der Waal interactions and coordination of Ca^{2+} (Jarvis 1984). This requires a stretch of approximately 10 unesterified GalA residues in order to be initiated (Daas *et al.*, 2001).

Previously an egg-box like structure was proposed to be the conformation of the Ca^{2+} crosslinked HG (Grant *et al.*, 1973). In the egg-box model anti-parallel chains were positioned so that the GalA residues from the separate chains were placed perpendicular to each other. Coordinating Ca^{2+}'s were then placed in the cavities between the two chains. Modeling studies suggest that an antiparallel structure with a distortion between the two chains and the Ca^{2+} placed closer to one of the chains is more energetically favorable compared to the egg-box structure. This could indicate that the egg-box model is not a correct model for Ca^{2+} induced HG dimerazation (Braccinni and Perez 2001). Methylation of HG weakens Ca^{2+} induced interactions and a too high degree of methylation disrupts Ca^{2+} induced interactions completely (Braccinni and Perez 2001). Other decorations of the HG seem also to disrupt Ca^{2+} mediated interactions. Acetylated HG cannot form Ca^{2+} mediated interactions (Pippen *et al.*, 1950) and addition of Xyl to C-3 of GalA as in XGA disrupts hydrogen bonding and introduce sterical hindrances (Own observation based on model from Braccinni and Perez 2001). Highly methylated HG can also form crosslinks though these crosslinks are based on hydrogen bonding and hydrophobic interaction between the methyl groups. The crosslinks can only be established under special conditions with pH < 3.5 to ensure protonation of carboxy groups, to suppress ionic hindrances and with a dry matter content > 50 %, to decrease the water activity and thereby increase hydrophobic interactions (Thakur *et al.*, 1997). Acidification of the cell wall has been proposed to be one of the main factors for initiating cell elongation (Hager *et al.*, 1971). But even during this acidification the pH is unlikely to be below 4 (Rayle 1973). This suggests that the conditions required for interactions between highly methylated HGs do not occour in the cell wall. Feruloylated arabinan and galactan was first reported to be present in spinach (Fry 1982). Later this was also found in sugar beet and it appears to be common for species in the *Amaranthaceae* including the agriculturally important sub family *Chenopodioideae*, which include spinach and sugar beet (Rombouts and Thibault 1986, Guillon and Thibault 1989, Clausen *et al.*, 2004). A monoclonal antibody against ferulyolated galactan has also been raised (Clausen *et al.*, 2004).

Ferulated arabinan has been suggested to be present in *Commelina communis* a commelinoid monocot, though it was not shown ambiguously that the ferulic acid did not originate from ferulyolated xylan (Jones *et al.*, 2003). In regards to xylan it is known that ferulic acid can dimerize by oxidation and thereby crosslink two xylan molecules. This esterification has also been reported between feruloylated arabinan or between feruloylated galactan polymers (Levigne *et al.*, 2004, Ralet *et al.*, 2005).

RG-II can form borate esters, crosslinking two RG-II structures. The crosslink is mediated between the apiose in chain A to a second chain A apiose in another RG-II molecule (Ishii *et al.*, 1999). Formation of dimeric RG-II is formed at pH > 3 (O'Neill *et al.*, 1996) and stabilized by divalent cations (Kobayashi *et al.*, 1999).

The above described interactions between pectin domains, with the concomitant formation of the pectin gel in the cell wall, are in line with the acknowledged function of pectin as a matrix component in the cell wall. If pectin should be able to modulate the mechanical properties of the cell wall, interactions with the hemicellulose-cellulose network could be essential. These interactions could confer stress between different hemicellulose and cellulose polymers modulation the plasticity of the cell wall. Pectin-cellulose interactions have been shown to be possible in *in vitro* studies. It was shown that arabinan and galactan, especially chains with a low degree of decoration, can interact with cellulose forming pectin-cellulose aggregates (Zykwinska *et al.*, 2005). An interesting aspect of this interaction study is that cellulose derived from cell wall had a larger binding capacity then the processed Avicel cellulose indicating the biological relevance.

Interactions between pectin and xyloglucan have also been documented. The best documented of these findings are based on rose suspension culture cell. In this work a fraction of purified xyloglucan proved to be anionic. Extensive studies lead to the conclusion that the pectin and xyloglucan was linked both via hydrophobic interactions and via covalent linkage. Which of the pectin domains that are linked to xyloglucan was not shown unambiguously but results indicated that arabinogalactan was involved. The linkage between pectin and xyloglucan was probably glycosidic, forming between the reducing end of xyloglucan and the nonreducing end of arabinogalactan (Thompson and Fry 2000).

In vitro biosynthesis of galactan was shown to lead to apparent covalent linkage to xyloglucan complementing the results from rose suspension cell (Abdel-Massih *et al.*, 2003). The same experiment could be interesting to perform, synthesizing arabinan instead of galactan to see if the same apparent covalent interaction could be established between arabinan and xyloglucan. Unfortunately UDP-Ara*f* is not available in a radioactive form. An in vitro study of binding of pectin to xyloglucan has also proved successful in showing interactions between the two polymers. The interaction was pH dependent with a maximum around pH three. It was hypothesized that the interactions had a role in cell wall assembly during cell elongation (Rizk *et al.*, 1999). As reported above the pH in the cell wall do not seem to be below four during cell elongation indicating that the interactions found has a minor biological significance (Rayle 1973).

Function of pectin *in muro*

Whether the *in vitro* studies presented in the previous paragraph can be extrapolated to also apply *in muro* can be debated. In the cell wall, pectin account for 30-50% of the dry matter and the water content is low. In *in vitro* studies the pectin concentration is around 1-5 % and the water content is high. Investigation into the function of pectin *in muro* is therefore best done using intact cell wall surrounding living cells, but technical hindrances can be insuperable.

Quasimodo1 (*qua1*) is a mutant with lowered amount of HG in the cell wall (Bouton et al 2002). This decrease in HG content leads to smaller plants and bulging of the epidermal cell layers. The bulging was suggested to be due to reduced cell to cell adhesion in the epidermal cell layers. Callus of *qua1* root cells have a reduced cell to cell adhesion supporting that HG is involved in cell to cell adhesion (Leboeuf *et al.*, 2005). Studies on wild type suspension culture cells also point towards a correlation between HG and cell adhesion (Leboeuf *et al.*, 2004).

In the tomato mutant *cnr*, ripe tomato pericarp is stronger, less swollen and less adherent compared to wild type (Orfila *et al.*, 2001). Sugar composition analysis of the cell wall did not show any gross alterations.

Electron energy loss spectroscopy, a method capable of measuring the calcium binding capacity at high resolution, was used for investigating the properties of the cell wall and the middle lamella. In both the cell wall and middle lamella, a significantly reduced calcium binding capacity was observed. Further immunochemical analysis showed that in *cnr*, a subset of water soluble HG had altered methylation and consequently disrupted calcium binding capacity.

The localization of low methylated HG, which can form Ca^{2+} interactions, near and in the middle lamella and at cell corners is in good agreement with its proposed role in cell to cell adhesion. Highly methylated HG has not been reported to be involved in modulating the mechanical properties of the cell wall and its function in the cell wall may be in the role as matrix polymer.

Arabinan and galactan have also been anticipated to be involved in cell to cell adhesion and in modulating the mechanical properties of the cell wall. The molar ratio of Ara to Gal in pectin from carrot callus cell walls has been shown to correlate with the cell cluster size, possibly reflecting different degrees of cell to cell adhesion (Kikuchi *et al.*, 1996). Galactan has been related with changes in mechanical properties of the cell wall e.g. rigidity and tissue texture (McCartney *et al.*, 2000, McCartney and Knox 2002, Smith *et al.*, 2002). In a T-DNA mutagenesis experiment with tobacco leaf material, the *nolac-h14* mutant was identified based on its loosely attached cells. *Nolac-h14* was shown to have a reduced amount of arabinan in its cell wall (Iwai *et al.*, 2001). Abnormal deposition in cell walls has been found associated with large intercellular spaces in the pericarp of the *Cnr* tomato mutant (Orfila *et al.*, 2001). However, the observations in Iwai *et al.*, 2001 and Orfila *et al.*, 2001 do not unambiguously correlate arabinan and galactan with cell to cell adhesion as HG was also affected in the mutants.

Arabinan has also been proposed to be involved in modulating the mechanical properties of the cell wall by acting as spacers for adjacent HG domains so Ca^{2+} induced HG dimers could not form (Jones *et al.*, 2003). If epidermal guard cells were closed by placing them in darkness, followed by incubation with arabinanase, subsequent opening induced by fusicoccin was limited. If PME was included along with arabinanase in the incubation of the guard cells, the guard cells opened even less indicating the involvement of formation of Ca^{2+} induced HG dimerasation.

If the guard cells were treated with ferulic acid esterase (FAE), an enzyme that cleaves the ester bond linking the ferulic acid to Ara, a decrease in the opening of the guard cells was also observed. The ferulic acid was suggested to be located on arabinan, but it was not shown unambiguously and could possibly also originate from feruloylated xylan. That galactan and arabinan are not as important as HG in facilitating cell to cell adhesion and possibly also in modulating mechanical properties can be suggested based on the lack of visual phenotype on galactan and arabinan deficient plants (Sørensen *et al.*, 2000, Skjøt *et al.*, 2002, Harholt *et al.*, 2006, Chapter 7).

RG-II, with its borate diester crosslink, is extremely important for normal cell growth and cell wall functionality. Plants grown in a low boron environment has altered cell wall plasticity and plants with reduced ability to form dimeric RG-II have a dwarfed phenotype and abnormal growth of leaves (Findeklee and Goldbach 1996, O'Neill *et al.*, 2001). Mutants with altered sugar composition in RG-II also show abnormal visual phenotype. The *mur1* mutant is impaired in synthesis of GDP-Fuc and as a consequence the Fuc and 2-O-Me-Fuc present in the RG-II is substituted with Gal and 2-O-Me-Gal instead, respectively (Bonin *et al.*, 1997, Reuhs *et al.*, 2003)(Figure 3). As Fuc and Gal only differ at the C-6 this could be seen as a minor alteration in RG-II but the mutant plant has the same phenotype as plants grown under low boron regimes (Reiter *et al.*, 1997). It was later shown that the formation of RG-II dimer is reduced two fold, indicating the significance of correct RG-II dimer formation (O'Neill *et al.*, 2001). In the *nolac-H18* mutant, which is lacking the GlcA and terminal Gal in chain A in RG-II, suspension cell cultures had showed abnormal growth with enlarged cells and apparent reduced cell to cell adhesion (Iwai et al 2002)(Figure 3). That RG-II is involved in cell to cell adhesion is not certain as the poor cell to cell adhesion could be a pleiotropic effect resulting from the altered cell wall. Localisation of RG-II in the cell wall indicate that RG-II is not involved in cell to cell adhesion as it is mainly located within the cell wall and not in the middle lamella (Matoh *et al.*, 1998). Field emission scanning electron microscopy pictures of the rupture surface of torn *mur1* hypocotyls also indicate that RG-II is not involved in cell to cell adhesion as the middle lamella is not exposed (Ryden *et al.*, 2003).

Direct measurement of stress on the cell wall

A plant is subjected to many mechanical stresses daily. Gravity is omnipresent for all plants, with perhaps the exception of plants growing submerged in water. In addition to the static stress from gravity more variable stresses e.g. wind also affect the plant. Internal stresses from turgor and negative pressure in the xylem also need to be endured by the cell wall. Some of these stresses can be mimicked *in vitro* and the force required for a given stress can be measured. Extracting pectin from the cell wall and then make it form a gel, in order to measure how the gel reacts to physical stress, do not take the interaction with other polymers and the whole cell wall architecture into account. Therefore measurements of physical stresses on intact plant tissue appear to be the better choice for physical stress experiments. From studies on mutants with alterations in a specific polymer one might deduce mechanical properties of the lacking polymer by comparing mutant with wild type plants.

One such approach uses potato tubers reduced in either arabinan or galactan content as model tissue in order to investigate the mechanical properties of the resulting cell wall (Ulvskov *et al.*, 2005). Cylinders of perimedullar potato tissue were compressed uniaxial and the force-deflection curves were recorded. Furthermore, stress-relaxation experiments were performed by shear of potato tuber cylinders.

Tubers with low galactan content had a lower strain to failure point and the force needed to reach failure point was lower. From stress-relaxation experiments the authors deduced that the tubers with reduced galactan had a faster relaxation rate in the cell wall. These differences were explained by decreased capability to transmit stress to cellulose and/or a reduced water conductance.

Reduction in arabinan led to a wall that broke slightly earlier and required less force before breakage. Strain-rate investigations indicated that the loss of arabinan influenced the polymer interactions making the wall more rigid. This observation correlated with the suggested model of arabinan function in guard cells (Jones *et al.*, 2003).

Another method for applying and measuring stress was presented in Ryden *et al.*, 2003 using *Arabidopsis* hypocotyls. The *mur1* and *arad1* mutant were tested using this method and the physical properties measured (Ryden *et al.*, 2003, Ryden Unpublished). Both *mur1* and *arad1* hypocotyls were weaker and less stiff.

The failure strain was not altered significantly in either of the mutants. The variation in the failure strain data sets are large and subtle changes could be lost due to this. In *mur1* the alterations were distinct compared to *arad1* where the changes were more subtly. But in both cases the alterations indicate that RG-II and arabinan are involved in cross linking the load bearing network in the cell wall. RG-II is well known as cross linker in the cell wall so this result is as expected. That arabinan can interact with cellulose has previously been suggested and the results obtained in the mechanical studies of *arad1* hypocotyls indicate that this interaction is of biological relevance (see also paragraph on intra and intermolecular interactions) (Zykwinska *et al.*, 2005, Ryden Unpublished).

The mechanical properties assigned to galactan and arabinan in Ulvskov *et al.*, 2005 was partly observed when the cell wall was stressed by compressing the cells. When the cell is compressed the turgor pressure increases, leading to stretching of the cell wall. The stretching of the cell wall is equally distributed so that the cell wall polymers move apart with the same relative distance to each other. Figuratively speaking, it could be compared to compressing a dotted balloon, where the dots then move apart with the same relative distance to each other. The force applied to the cell wall by pulling the hypocotyls, as in Ryden *et al.* 2003 and Ryden Unpublished, lead to an unequal stretching of the cell wall polymers compared to each other. Again using the dotted balloon as an example, the consequence of pulling is that some dots move closer and other away from each other. This difference could explain the discrepancy in the results obtained from arabinan deficient tubers and from *arad1* hypocotyls as the cell wall itself is directional in its structure with the cellulose microfibrils arranged parallel in different layers. It could be interesting to test galactan deficient *Arabidopsis* hypocotyls to see if conflicting results, compared to galactan deficient potato tubers, were obtained as in the case of arabinan. Unfortunately a galactan deficient *Arabidopsis* mutant has not yet been identified.

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Chapter 2

This chapter comprises a review on pectin biosynthesis. *In vitro* methods for assaying glycosyltransferase activity is discussed. Different approaches in the identification of novel glycolsyltransferases as well as the current work on identification of novel glycosyltransferases are presented.

Biosynthesis of pectin

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Abstract

Pectin consists of a group of acidic polysaccharides that constitute a large part of the cell wall of plants. The pectic polysaccharides have a complex structure but can generally be divided into homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan. These polysaccharides appear to be present in all cells but their relative abundance and structural details differ between cell types and species. Pectin is synthesized in the Golgi vesicles and its complexity dictates that a large number of enzymes must be involved in the process. The biosynthetic enzymes required are glycosyltransferases and decorating enzymes including methyltransferases, acetyltransferases, and feruloyltransferases. Biochemical methods have been mostly unsuccessful in leading to the identification of the enzymes involved in pectin biosynthesis. However, recent functional genomics and mutant studies have allowed the identification of several biosynthetic enzymes involved in making different parts of pectin. Unambiguous evidence of enzyme function has been obtained for a galacturonosyltransferase, involved in making homogalacturonan, and for two xylosyltransferases, involved in making a side chain of rhamnogalacturonan II, since their activities have been confirmed by analysis of the heterologously expressed proteins. Strong circumstantial evidence has been obtained for a putative glucuronosyltransferase involved in making rhamnogalacturonan II, a putative arabinosyltransferase involved in making arabinan, and a putative xylosyltransferase involved in making xylogalacturonan. In several other cases, enzymes have been identified as involved in making pectin but due to ambiguity in the cell wall compositions of mutants and lack of direct biochemical evidence their specific activities are uncertain.

Abbreviations

CAZy, Carbohydrate Active Enzyme Database; GT, glycosyltransferase; HGA, homogalacturonan; RGI, rhamnogalacturonan I; RGII, rhamnogalacturonan II; XGA, xylogalacturonan; WT, wild type

The cell wall

All plant cells are surrounded by a cell wall consisting of polysaccharides as the most abundant component, proteins and sometimes lignin. The cell walls have several very important functions in the plant: they allow the plant to stand upright, they give the cells form, they mediate interactions between cells, and they provide a barrier against attack from herbivores and disease agents. The cell wall consists of polymers that are deposited outside the cell but nevertheless they are by no means a dead and inert substance. In contrast, the walls are plastic and polysaccharides are turned over and reformed continuously in the primary wall (Gibeaut and Carpita 1991, Inoue et al. 1997). Hence, cells are able to expand their length to more than hundred times the initial length although they are encased in a wall of strong cellulose microfibrils and matrix polysaccharides.

The polysaccharides of the wall are usually divided in cellulose, hemicellulose and pectin. Hemicellulose is a heterogeneous group of polysaccharides with xyloglucan and xylan being the most abundant in most cells. Most plants have so called Type I primary walls where xyloglucan is the most abundant hemicellulose. In contrast, grasses and other commelinoid species have Type II primary walls with arabinoxylan as the most abundant hemicellulose. Grasses also have a unique feature of hydroxycinnamate esters bound to the arabinoxylans. Pectin is highly heterogeneous, but is a relatively well defined type of polysaccharide characterized by the high content of galacturonic acid. Pectin occurs in all cells and typically constitutes about 30% of Type I primary walls. Secondary walls and Type II primary walls normally have a much lower content of pectin. Proteins are important components of the cell wall and comprises both structural proteins and enzymes, e.g. extensins, arabinogalactan proteins, expansins, and polysaccharide hydrolases. For a review on the structure and composition of cell walls in flowering plants see Carpita and Gibeaut (1993).

The structure of pectin

Pectin can be extracted from cell walls by a variety of methods, including hot acid, mild alkaline treatment and chelating compounds. Enzymes that degrade parts of the pectin can also be used to release pectin. The extracted pectin can be fractionated into homogalacturonan (HGA), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII) and xylogalacturonan (XGA) (Willats et al. 2001). Apparently, these polymers are covalently linked to each other, but it has been very difficult to get a clear picture of how the different pectic polysaccharides are connected and several models exist (Vincken et al. 2003).

A highly schematic model of pectin is shown in Fig. 1. HGA is a homopolymer of α-1→4-linked GalA residues which are often methylesterified on C6. HGA can be acetylated on C2 or C3 of the GalA residues, but the degree of acetylation varies a lot between species. RGI consists of a backbone with the disaccharide [α-1→4-GalA-α-1→2-Rha] as the basic repeating unit. The rhamnose residues are often substituted with galactan, arabinan or arabinogalactan side chains, and the GalA residues can be acetylated as in HGA. In some species the arabinose and galactose residues in RGI side chains can be substituted with ferulic and coumaric acid esters. RGII is a very complex polysaccharide but its structure appears to be remarkably conserved in all vascular plants (Matsunaga et al. 2004). The name RGII is misleading since its backbone is not rhamnogalacturonan but a short stretch of HGA substituted with four different sidechains. RGII consists of a total of 12 different monosaccharides in more than 20 different linkages. The residues in RGII includes some monosaccharides that are rarely found in other polysaccharides: D-apiose, 3-C-carboxy 5-deoxy-L-xylose (L-aceric acid), 2-*O*-methyl L-fucose, 2-*O*-methyl D-Xylose, L-Galactose, 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), and 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo). RGII exists predominantly as dimers that are covalently cross-linked by borate diesters. Immunocytochemical studies using antibodies raised against RGII suggested the presence of RGII in primary walls of all cells in a wide range of species except that little RGII was apparent in the middle lamella (Matoh et al. 1998). XGA is similar to HGA except that it is substituted with single ß-1→3-Xyl residues or such residues substituted with a few additional ß-1→4-Xyl residues (Schols et al. 1995). XGA has been reported especially in reproductive tissues, but this type of pectin is probably present in all tissues (Zandleven et al., submitted).

Pectin is synthesized by glycosyltransferases in the Golgi vesicles
Determination of enzyme activities in membrane fractions and labeling of tissues with antibodies raised against pectin epitopes have shown that pectin biosyntheis takes place in the Golgi vesicles (Staehelin and Moore 1995, Willats et al. 2001, Ridley et al. 2001). In addition, some of the biosynthetic enzymes (RGXT1, ARAD1, and XGD1, see below) have been shown to be located in the Golgi vesicles when expressed as fluorescent fusion proteins (Egelund et al., in preparation; J.K. Jensen, Y. Sakuragi, and H.V. Scheller, unpublished data). However, it cannot be excluded that some early stages of pectin biosynthesis takes place in the endoplasmic reticulum or that some maturation steps take place at the plasma membrane or even in the cell wall.

Due to the large complexity of pectin it is clear that a large number of biosynthetic enzymes must be required for its synthesis. Mohnen (1999) has estimated that 54 different activities are required based on the assumption that each enzyme has a specificity that is defined by the glycosyl linkage formed between each two unique glycosyl residues. Most of the predicted enzymes are glycosyltransferases (GTs) that transfer one glycosyl residue from a nucleotide sugar donor to the non-reducing end of an oligo- or polysaccharide acceptor. The majority of the GTs are likely to be type II membrane proteins with an N-terminal transmembrane anchor and a catalytic domain in the Golgi lumen. However, multi-membrane spanning GTs are also present in the Golgi vesicles (e.g. mannan synthase, Dhugga et al. 2004) and it is possible that some are involved in pectin biosynthesis. Transglycosylases that transfer whole oligosaccharide chains from one polysaccharide to an acceptor polysaccharide are well known in starch and xyloglucan biosynthesis. While no transglycosylase activity acting on pectin has been reported, it is conceivable that such enzymes exist. In addition to GTs and possible transglycosylases, the biosynthesis of pectin requires several different acetyl-, methyl-, and feruloyltransferases. Acetyl- and methyltransferases are dependent on acetyl-CoA and SAM, respectively (Pauly and Scheller 2000, Bruyant-Vannier et al. 1996), while the donor substrate for feruloylation is not known.

Pectin biosynthesis has been detected in vitro

Pectin biosynthesis has mostly been investigated by incorporation of radioactive sugars from nucleotide sugar substrates into polysaccharide-products in membrane fractions from different plant sources. Numerous investigations of pectin biosynthesis in vitro have been carried out over the past 40 years but a thorough treatise of the extensive literature is beyond the scope of this review. In the early studies, it was very difficult to characterize which products that were actually formed and, in spite of much work, none of the specific enzymes were identified. More recently some hydrolases that cleave specific sites in pectic oligosaccharides became available and this has made it possible to analyze the products in more detail. Also, development of column chromatography techniques, which can separate oligosaccharides with high resolution, has helped to analyze the products.

Villemez et al. (1965) demonstrated biosynthesis of HGA by *Phaseolus aureus* membranes already in 1965. An important subsequent advance in the study of HGA synthesis was the development of acceptor dependent assays on exogenous oligosaccharide acceptors of well-defined structure (Doong and Mohnen 1998).

When endogenous acceptor substrates are used, the products are very heterogeneous and the result of the activity of several enzymes that act simultaneously. A GT activity that transfers a galacturonosyl residue from UDP-^{14}C-galacturonic acid specifically onto α-1→4-linked oligogalacturonides was demonstrated in membranes prepared from tobacco (Doong et al. 1995, Doong and Mohnen 1998). The enzyme activity could be solubilized and this work eventually led to identification of the biosynthetic enzyme GAUT1 (see below). GT activity that elongates galactan side chains has been demonstrated in membrane fractions from different plant sources. In early studies, products after incorporation of ^{14}C-galactose from UDP-^{14}C-galactose were analyzed by solubility into water and/or alkali (e.g. McNab et al. 1968). Geshi et al. (2000) demonstrated a GT activity in membranes prepared from potato suspension cells that synthesized galactan onto endogenous oligosaccharide-acceptors which could be digested by an endo-hydrolase that specifically cleaves β-1→4-galactooligosaccharides. Peugnet et al. (2001) and Geshi et al. (2002) solubilized a GT activity from flax and potato suspension cells, respectively, that transfers ^{14}C-galactose onto exogenously applied RGI-acceptors and demonstrated that the synthesized products could be digested by a series of hydrolases, which cleave galactan side chains and the RGI backbone. The RGI-acceptors used in these studies were heterogeneous, but Ishii et al. (2004) demonstrated a GT activity in membranes prepared from mung bean hypocotyl that transfers up to 8 galactosyl residues onto the non-reducing end of well-defined and fluorescence-labeled galactooligosaccharides. Thus, several laboratories have demonstrated GT activities that elongate galactan side chains, but none have yet reported a GT activity that initiates the side chain by transferring the first galactose residue onto the RGI backbone. Apparently, the membranes that are active in elongating galactan side chains do not incorporate galactose into RGI oligosaccharides in the absence of any substitutions (Geshi et al. 2002; Ishii et al. 2004). An arabinosyltransferase activity possibly involved in biosynthesis of pectin was first reported in mung bean (Odzuck and Kauss 1972). Later work using membrane preparations from *Phaseolus vulgaris* yielded a product that was degraded by a pectinolytic enzyme preparation (Bolwell and Northcote 1981). An assay for arabinosyltransferase dependent on exogenous acceptors was eventually reported by Nunan and Scheller (2003). A single ^{14}C-arabinopyranose residue from UDP-ß-L-^{14}C-arabinopyranose was transferred onto α-(1→5)-linked arabinooligosaccharides in membranes prepared from mung bean hypocotyls.

Ishii et al. (2005) used fluorescence-labeled arabinooligosaccharide acceptors and demonstrated that the GT activity in mung bean hypocotyls transfers the single arabinopyranose residue onto O-3 of the non-reducing terminal arabinofuranose. The same group also demonstrated a GT activity that transfers a single arabinopyranose onto galactooligosaccharides (Ishii et al. 2005b). Thus, arabinopyranosyl transferase activity has been reported by different groups. However, in plant polysaccharides most of the arabinose residues are present in the furanose form and the biological relevance of the arabinopyranosyltransferase activity is therefore not clear. In many bacteria a galactofuranosyl transferase is present that uses UDP-galactofuranose as the substrate. The UDP-galactofuranose is produced from UDP-α-D-galactopyranose by the action of a mutase and the bacterial mutase has been shown also to convert UDP-ß-L-arabinopyranose to UDP-ß-L-arabinofuranose (Chang and Liu 2001). This has caused speculation that a similar activity is present in plants but no obvious homologs of the bacterial mutase has been found. However, very recently Ishii and coworkers have shown that membranes from mung bean hypocotyls contain a very active transferase activity that can transfer successive arabinofuranosyl residues from UDP-ß-L-arabinofuranose onto arabinooligosaccharides (Konishi et al. 2006). Therefore, although a plant mutase has not been reported it seems highly likely that such an activity should be present in plants and required not only for arabinan biosynthesis but for all arabinofuranose incorporation in plants.

Biosynthetic enzyme activities that modify pectic polysaccharides, e.g. pectin methyltransferases (Bruyant-Vannier et al. 1996, Goubet et al. 1998) and O-acetyltransferases (Pauly and Scheller, 2000) have also been demonstrated.

Functional genomics

With the exception of the identification of GAUT1 (see below) the biochemical studies of pectin biosynthesis have not resulted in identification of the enzymes involved. This seems to be due to a combination of relatively unstable enzymes and very low amounts of these enzymes. However, the progress in genomics has opened new methods for identification of the GTs and other biosynthetic enzymes. GTs belong to the carbohydrate active enzymes which are compiled in the CAZy database at http://afmb.cnrs-mrs.fr/CAZY (Cutinho and Henrissat 1999). The database has the GTs organized in 83 different families, 39 and 40 of which are represented in rice and Arabidopsis, respectively. The families are based on the Pfam database of multiple alignments of protein domains or conserved protein regions.

In spite of the sequence similarities it is very difficult to predict the specificity of the different GTs because the nucleotide sugar and acceptor substrates are not well conserved within each family. The anomeric configuration of the glycosidic bound created by the GT can either retain or invert the configuration from the donor sugar. Thus, inverting GTs produce β-linkages with D-sugars and α-linkages with L-sugars while retaining GTs produce α-linkages with D-sugars and β-linkages with L-sugars. Apparently, whether enzymes are inverting or retaining is conserved within each family. However, although this rule has seemed to hold, one cannot take for granted that it will always be true as e.g. seen for the retaining GT27 family, which was originally part of the inverting GT2 family due to sequence similarities (Breton et al. 1998). A specific pattern in the amino acid hydrophobicity of a few residues in the active site appears as the determining factor towards either retaining or inverting activity of the enzyme (Rosén et al. 2004).

CAZy is a very useful resource for the study of cell wall biosynthetic enzymes, but a very important question is how comprehensive it is. Some plant GTs are probably not classified in CAZy simply because no homologs have yet been identified in any species. In fact among several putative non-CAZy GTs identified in the Arabidopsis proteome by bioinformatics (Egelund et al. 2004), two of them turned out to be α-1→3-xylosyltransferases involved in the biosynthesis of RGII (Egelund et al. unpublished). These two GTs and other related sequences were later classified to a new family in CAZy (GT77) according to Egelund et al. (2004). While there is such an example, we find it likely that most *Arabidopsis* GTs are in fact represented in CAZy. If many non-CAZy GTs were present, it would be expected that cell wall mutants identified by forward genetics would often lead to identification of new GT families. While this was true some years ago, it is no longer the case. Given that only a very small fraction of all the enzymes required for synthesis of the wall have been identified, most of the enzymes in CAZy are of course annotated as 'unknown protein' or with a very unspecific designation. In principle, the pectin biosynthetic enzymes should have sequences for targeting to the Golgi vesicles. Unfortunately, reliable bioinformatic methods for predicting Golgi localization are not available, but at least targeting to the secretory pathway can be predicted with some confidence.

With the availability of microarray analyses of the *Arabidopsis* transcriptome, bioinformatics can be used to identify enzymes potentially involved in pectin biosynthesis. One approach is to compare control or wild type (WT) expression levels to treated plants or mutants.

Thus, Manfield et al. (2004) used isoxaben, which is an inhibitor of cellulose biosynthesis and leads to increased biosynthesis of hemicelluloses and pectin, to probe the induced changes in transcript profiles and identify differently expressed genes. This lead to the discovery of at least one putative non-CAZy GT. Several genes encoding enzymes known to be involved in pectin biosynthesis, e.g. QUA1 and GAUT1, were upregulated in isoxaben-habituated cells. In a more indirect approach, the expression profiles of genes in a large number of experiments can be compared to identify coregulated genes. Thus, Persson et al. (2005) utilized the fact that some *CESA* genes are specifically involved in secondary cell wall cellulose biosynthesis to find genes in the publicly available Arabidopsis microarrays that have similar expression patterns. This lead to three candidate GTs located in GT8, GT43 and GT47. The GT47 member, *FRA8*, has since been found to be involved in glucuronoarabinoxylan biosynthesis showing the potential by the bioinformatics approach using experimental data that is freely available (Zhong et al. 2005).

With the oncoming of the full genomic sequence of other plants, including the already available rice and poplar, large scale comparable genomic work could be initiated. Species such as *Chlamydomonas reinhardtii* and *Physcomitrella patens* represent evolutionary more distinct groups that are about to be fully sequenced. The knowledge of cell wall composition in these species is unfortunately still very limited but potentially these genome sequences will be a very useful resource for comparative genomics and prediction of biosynthetic enzyme activities.

Genes and proteins involved in biosynthesis of HGA

Screening of mutants for growth defects have lead to identification of several GTs involved in cell wall biosynthesis but a direct involvement in pectin formation has only been implied in a few cases. The *quasimodo1* (*qua1*) mutant in Arabidopsis showed deficiency in cell adhesion and stunted growth (Bouton et al. 2002). Analysis of the mutant showed several cell wall defects but a particularly large decrease in HGA. Cloning of the mutated gene demonstrated that it was a GT in CAZy Family 8 – a family of enzymes predicted to be retaining. Given that the backbone of HGA is synthesized by a retaining galacturonosyl transferase, it was hypothesized that QUA1 could be such an enzyme. Analysis of biosynthetic enzyme activities showed that HGA biosynthesis was indeed decreased in the mutant but so was xylan synthesis (Orfila et al. 2005). Due to these pleiotropic effects and absence of direct biochemical evidence it has not been possible to conclude if QUA1 is a galacturonosyl transferase.

However, Mohnen and coworkers have successfully purified an HGA- galacturonosyl transferase activity from Arabidopsis and identified a GT8 member as responsible for the activity (Sterling et al. 2006). This activity was confirmed after heterologous expression of the enzyme in human embryonic kidney cells. The enzyme has been designated GAUT1 (GAlactUronic acid Transferase 1). Mohnen and coworkers purified a homolog, GAUT7, together with GAUT1, but could not demonstrate enzymatic activity for this protein. The recent paper on GAUT1 is a landmark since it is the first publication where the identification of an enzyme involved in pectin biosynthesis is reported together with biochemical evidence for the biochemical function.

GAUT1, GAUT7 and QUA1 belong to a subgroup of GT8 with 15 members in Arabidopsis (Fig. 2). Another subgroup with 10 members is similar to the GAUT group and has been designated GATL (GAlacturonic acid Transferase Like) (Sterling et al. 2006). The GATL subgroup includes the *PARVUS* gene, which is known to be important for cell wall development (Lao et al. 2003) but has not been directly linked to pectin biosynthesis. Sterling et al. (2006) suggest that all the GAUTs and GATLs are galacturonosyl transferases involved in pectin biosynthesis (Sterling et al. 2006). Future work will show if they differ in their specificity for incorporating galacturonic acid residues in the different parts of pectin. They may also turn out to differ more in their expression patterns than in enzymatic specificity. Forward genetics has also led to identification of the *quasimodo2* mutant which resembles *qua1* in the Fourier transformed IR spectrum of the cell wall (Mouille et al. 2003). Cloning of the corresponding gene showed that it is a putative SAM-dependent methyltransferase (G. Mouille, personal communication). Hence, these data suggest that QUA2 is a pectin methyl transferase.

Genes and proteins involved in biosynthesis of RGI and XGA

CAZy family GT47 is very large in plants and contains members without orthologs in other organisms (Fig. 3). Furthermore, GT47 has already been shown to contain several cell wall biosynthetic enzymes involved in xyloglucan and glucuronoarabinoxylan biosynthesis (Madson *et al.* 2003, Li *et al.* 2004, Zhong et al. 2005). Screening of *Arabidopsis* knock-out mutants in GT47 led to identification of ARAD1 (ARABINAN DEFICIENT 1), a GT involved in arabinan biosynthesis (Harholt et al. 2006). The knock-out mutants contain only 25% and 50% of the WT arabinose in leaf and stem, respectively. Immunolabelling and cell wall fractionation lead to the discovery that *arad1-1* contained shorter arabinan side chains in the RGI.

Cell wall analysis of T-DNA knock out mutants of ARAD1's nearest homolog, tentatively designated ARAD2, showed no differences compared to WT (C. Søgaard, J. Harholt and H.V. Scheller, unpublished results). Transformation of *arad1* with *CaMV35S:ARAD1* led to complementation of the arabinan deficient phenotype, but the highly elevated levels of ARAD1 in the plants did not lead to increased arabinan content (Harholt et al. 2006). The same lack of increased amount of arabinan applies when WT was transformed with *CaMV35S:ARAD1* (Harholt et al. 2006). This could indicate either a very tight regulation of enzyme activity or that another component is limiting, perhaps a subunit of a protein complex that includes the GT. Another possibility is that nucleotide sugar substrates are limiting. ARAD1 and ARAD2 are members of subgroup B of GT47. Mohnen (1999) has estimated that there should be 12 α-arabinofuranosyltransferases involved in pectin biosynthesis. However, if the specificity of the enzymes would depend only on the penultimate non-reducing sugar of the acceptor then only 6 α-arabinofuranosyltransferases would be required. Subgroup B of GT47 contains 8 members in Arabidopsis and it is thus conceivable that they encode the α-arabinofuranosyltransferases involved in pectin biosynthesis. However, much more biochemical studies are needed to clarify this and we cannot predict whether additional α-arabinofuranosyltransferases should be expected.

Galactan is the other major side chain of RGI besides arabinan. At this time there are no mutant studies or biochemical data pointing towards possible candidates for the synthesizing proteins.

Screening of GT47 mutants also led to discovery of a mutant deficient in XGA (Sørensen et al., in preparation). We have designated the mutant *xylogalacturonan deficient 1* (*xgd1*). XGA has been found to be present in *Arabidopsis* in a relatively low substituted form (Zandleven et al., submitted). In the *xgd1* mutant, XGA was almost completely absent. Transformation of the mutant with *CaMV35S:XGD1* restored the cell wall composition to the WT. The data suggest that the XGD1 protein encodes a ß-1,3-xylosyltransferase involved in attaching xylose to a HGA backbone.

Genes and proteins involved in biosynthesis of RGII

Three genes encoding GTs involved in RGII biosynthesis have been reported to date. *NpGUT1* was identified as the affected gene responsible for the phenotype in the *Nicotiana plumbaginifolia nolac-H18* mutant, which has impaired cell attachment in callus culture (Iwai et al. 2002).

The encoded NpGUT1 protein shows sequence similarity to animal glucuronosyltransferases and is classified to GT47 in the CAZy database (Fig. 3). Sugar composition of RGII isolated from *nolac-H18* showed almost complete lack of glucuronic acid and approximately 50% of RGII in the mutant was present as monomers whereas more than 95% of RGII in WT was present as dimers. In vitro studies confirmed that RGII from the mutant is less capable to form dimers compared to the WT RGII. Transfomation of the mutant with *CaMV35S::NpGUT1* complemented the cellular attachment phenotype, level of glucuronic acid, and ability of dimer formation in RGII. Thus, although demonstration of the enzyme activity has not been accomplished, NpGUT1 is most likely a glucuronosyltransferase that synthesizes the α-L-Gal*p*-(1→2)-ß-D-GlcA-(1→) part of side chain A of RGII.

Two other GTs involved in the RGII biosynthesis are RGXT1 and RGXT2. The two putative proteins were identified by a bioinformatics approach as members of a new group of plant specific GTs in Arabidopsis and classified to a new family of GT77 in the CAZy database (Egelund et al. 2004). The two highly homologous genes, *RGXT1* and *RGXT2*, which are clearly the result of recent gene duplication, were heterologouly expressed and demonstrated xylosyltransferase activity by transfer of D-xylose from UDP-D-xylose to L-fucose residues with α-glycosidic linkage (Egelund et al. in preparation). α-methyl fucose worked as the best acceptor among several compounds tested and NMR analysis confirmed the product as α-D-xylose-(1→3)-L-fucose. The only known structure containing α-D-xylose-(1→3)-L-fucose is in side chain A of RGII. The xylose in side chain A is methylated and is a diagnostic feature of this side chain. The level of methyl-D-xylose in the isolated RGII from T-DNA insertional mutants in *RGXT1* and *RGXT2* was slightly decreased in comparison to the WT RGII. The small effect suggest that the RGXT enzymes are redundant and largely complement each other. Because the two genes are closely linked it is not feasible to construct a double mutant. Nevertheless, RGII isolated from the different mutant lines worked well as acceptors for RGXTs and there was a good correlation between the reduction level of methyl-D-xylose and incorporation level of xylose in vitro. WT RGII did not function as acceptor. The results demonstrate that RGXTs are α-(1→3)-xylosyl transferases that synthesizes the methyl-α-D-xylose-(1→3)-L-fucose portion of side chain A of RGII.

How many pectin related GTs are there?

The research reviewed above has led to the identification of five different GT activities related to pectin biosynthesis (Table 1). As treated ealier by Mohnen (1999), a central question is how many GTs we can expect to be involved in pectin biosynthesis. The limited number of already identified GTs renders it difficult to make a good prediction based on these, but a simple way to estimate this for the cell wall biosynthesis as a whole is to compare the size of the different CAZy GT-families between plant and non-plant eukaryote species. Table 2 groups the 54 GT families in CAZy relevant for the listed plant and animal species in four different categories. A little less than half of the families contain comparable numbers of GTs for all six organisms in the table and a reasonable assumption is that they are largely involved in functions that are conserved between these species, i.e. functions unrelated to the cell wall. The plant dominating group of GT-families indicated in the table contains 17 individual families representing a total of 231 GTs for *Arabidopsis*, most of which are members of only five different families, GT4, GT8, GT31, GT47, and GT77, containing 148 GTs in total. The plant dominating GT-families have numbers of GTs that are at least 10 times larger when compared to the same families for the animals in the table. Because of the uniqueness of the plant cell wall, this overrepresentation of approximately 200 GTs is likely to a large extent to originate from GTs involved in plant cell wall biosynthesis.

The number of about 200 cell wall GTs poses some interesting questions when considering the chemical diversity of the plant cell wall polymers and the progress in the field so far concerning biosynthetic redundancy and GT specificity. In GT2, which contains the *CESA* and the *CSL* genes, the dominating hypothesis is that each subgroup is responsible for synthesizing a specific homopolymer, for instance cellulose for the ten Arabidopsis *CESA* genes, and (gluco)mannan for the seven *CSLA* genes. indicating a high degree of redundancy. Similarly, Sterling et al. (2006) proposed 25 GAUTs and GATLs in GT8 to be galacturonosyl transferases but the known linkages of galacturonic acid residues may only require 4 to 7 different transferase activities (Mohnen 1999).

Studies of xyloglucan biosynthesis have indicated that GTs may be quite specific since xylosylation at glucosyl residue 1, 2 and 3 of the repeating unit is not carried out by the same activity (Faik et al. 2002) and the further galactosylation at position 2 and 3 also requires separate enzymes (Madson et al. 2003). If a similar narrow specificity is common in pectin biosynthesis, the side chain generation in the different pectins would require much more than the about 40 different activities estimated by Mohnen (1999).

With about 60 GTs from GT2 and GT8 required for the backbone biosynthesis of cellulose, hemicellulose and galacturonan there are less than 150 GT candidates to cover all the side chain generation in pectin, the hemicelluloses, and possibly the synthesis of the glycosidic part of the AGPs and other plant-specific glycoproteins. The number of about 200 cell wall GTs therefore appears somewhat less than might be expected.

This apparent discrepancy may be explained by a considerable number GTs in plants that are so unique in their primary sequence that they have not been identified by the homology approaches used so far. As argued above, we do not find this very likely. Alternatively, the redundancy outside GT2 and GT8 may be very low, which is somewhat supported by the progress in GT47 that has not revealed substantial redundancy for these genes. Lastly, we propose that some GTs might work in a multifunctional way such that their acceptor specificities are affected by other proteins for instance by protein-protein interactions.

Are pectin biosynthetic enzymes organized in complexes?

In the major glycogenic pathways in mammals, such as *N*- and *O*-linked glycosylation, and in plants, such as cellulose and callose biosynthesis, protein-protein interactions act as an important organizing principle with regards to biosynthetic coordination, subcellular localization and direct regulation of enzymatic activity (Young, 2004; Doblin et al., 2002; Hong et al., 2001). Until now there has been no clear evidence that protein complexes are involved in biosynthesis of pectin or hemicellulose, but this probably reflects the very limited number of identified proteins and the few detailed studies. Overexpression of ARAD1 and XGD1in *Arabidopsis* did not lead to more arabinan or XGA, respectively (Harholt et al. 2006, J.K. Jensen and H.V. Scheller, unpublished). This could suggest that each of these proteins function in a biosynthetic complex and that the limited amount of the other components in such complexes result in the absence of overproducing phenotypes.

Future studies and needs

An essential step in the process of identification of pectin biosynthetic enzymes is the verification of the biochemical activity of the enzyme. This generally requires heterologous expression in order to obtain sufficient protein that is free from contaminating activities. Different eukaryotic expression systems have been tried all with low success rate. *Pichia pastoris*, insect cells using the baculovirus system, *Drosophila* cells, human embryonic kidney cells, and COS cells have all been used successfully, but none of these systems have proven to be generally applicable.

Transient expression in *Nicotiana benthamiana* is an alternative method to generate proteins in a plant system, which might have obvious advantages, but it is not yet clear if this system will be generally useable. Having solved the first problem of getting the protein expressed raises the next sometimes even greater challenge to sort out the assay conditions. To find the right combination of nucleotide sugar donor and acceptor substrate is a daunting task if the phenotype of mutants or sequence similarities does not give good hints about the expected activity. One possibility to overcome this is by using the 'free sugar assay' where a monosaccharide in very high concentration is used as acceptor with different combinations of nucleotide sugar donor (Brückner et al. 2001). This method was used in the initial studies to resolve the activity of RGXTs (Egelund et al. unpublished). Another potential problem could be that some enzymes simply cannot work as a single protein but must be in heteromeric complexes that would thus require the simultaneous heterologous expression of several proteins.

An obvious requirement for assaying activity is that the substrates for detecting enzymatic activities are available. Currently, we lack especially UDP-rhamnose but also apiose, aceric acid, Dha and Kdo are not available as donor nucleotide sugars. The enzymes catalyzing the conversion of UDP-glucose to UDP-rhamnose and UDP-glucuronic acid to UDP-apiose are known (Watt et al. 2004, Mølhøj et al. 2003) but the nucleotide sugars have not yet become commercially available. UDP-arabinopyranose is commercially available in a non-radioactive form from the Carbosource Service (http://www.ccrc.uga.edu/~carbosource/CSS_home.html) and can be produced in the radioactive form by a relatively simple procedure (Pauly et al. 2000). However, there are strong indications for the necessity of using UDP-arabinofuranose for in vitro studies (Konishi et al. 2006). So far, UDP-arabinofuranose can only be obtained by chemical synthesis and has only been produced in a non-radioactive form. For feruloyation we have no indication of what the activated donor may be, but feryloyl-CoA, feruloyl-glucose and feruloyl-quinate are all possible candidates (Obel et al. 2003) and in all cases they are not commercially available.

Biosynthetic enzyme assays require the simultaneous presence of both a donor and an acceptor substrate and hence there are an almost unlimited number of combinations to be tested for a protein with unknown activity. It would be a great advantage if it were possible to screen enzymes with just one substrate, e.g. for affinity to different nucleotide sugars without the need for presence of the acceptor.

In principle this may be possible by a range of different methods. GTs have been shown to bind to nucleotide sugars in the absence of acceptor (Perrin et al. 1999) and this may enable development of a method to screen GTs for the most likely donor substrates.

Although there are many challenges ahead, the recent breakthroughs hold promise that we will soon identify many of the enzymes involved in pectin biosynthesis. Determining how they interact with each other and how they are regulated to serve the needs of the organism is a tremendous challenge for the future.

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Tables

Table 1. Identified pectin biosynthetic enzymes

Enzyme designation	Monosaccharide transferred	Polysaccharide produced	Confirmed biochemical activity	Reference
GUT1	glucuronic acid	RGII		Iwai et al. 2002
QUA1	galacturonic acid?	HGA?		Bouton et al. 2002
RGXT1 RGXT2	xylose	RGII	X	Egelund et al. 2006[a]
ARAD1	arabinose	RGI		Harholt et al. 2006
GAUT1	galacturonic acid	HGA	X	Sterling et al. 2006
XGD1	xylose	XGA		Sørensen et al.[b]

[a]Unpublished data (submitted for publication)

[b]Unpublished data (in preparation).

Table 2. Number of GTs among higher eukaryotes and their comparable distribution within the different CAZy GT families[1]

	Common	Animal dominating	Plant dominating	CAZy GT1[2]	Total
CAZy Families	10, 13, 14, 16, 17, 21, 22, 24, 31 32, 33, 35, 41, 43, 50, 57, 58, 59, 64, 65, 66, 68	3, 6, 7, 11, 12, 18, 23, 25, 27, 29, 39, 49, 54, 76	2, 4, 5, 8, 19, 20, 28, 30, 34, 36, 37, 38, 47, 48, 61, 75, 77	1	
Arabidopsis thaliana	**88**	**4**	**231**	**121**	**444**
Oryza sativa (**japonica cultivar-group**)	**92**	**3**	**236**	**194**	**525**
Populus trichocarpa	**133**	**5**	**353**	**326**	**817**
Caenorhabditis elegans	**89**	**54**	**21**	**77**	**241**
Drosophila melanogaster	**58**	**32**	**17**	**36**	**143**
Homo sapiens	**87**	**91**	**27**	**24**	**229**

[1]CAZy glycosyltransferase families with no animal or plant members are not listed. The numbers in the table are the total number of annotated GTs in the four different categories of families. The data are from the CAZy database http://afmb.cnrs-mrs.fr/CAZY/ except for *Populus trichocarpa* (Geisler-Lee et al. 2006).

[2]CAZy Family 1 falls in between the Common and the Plant dominating categories. Family 1 is very large and in plants it is believed to represent non-membrane bound glycosyltransferases involved in secondary metabolism glycosylating various metabolic compounds, growth factors etc. For these reasons the family is listed as a separate category.

Figures

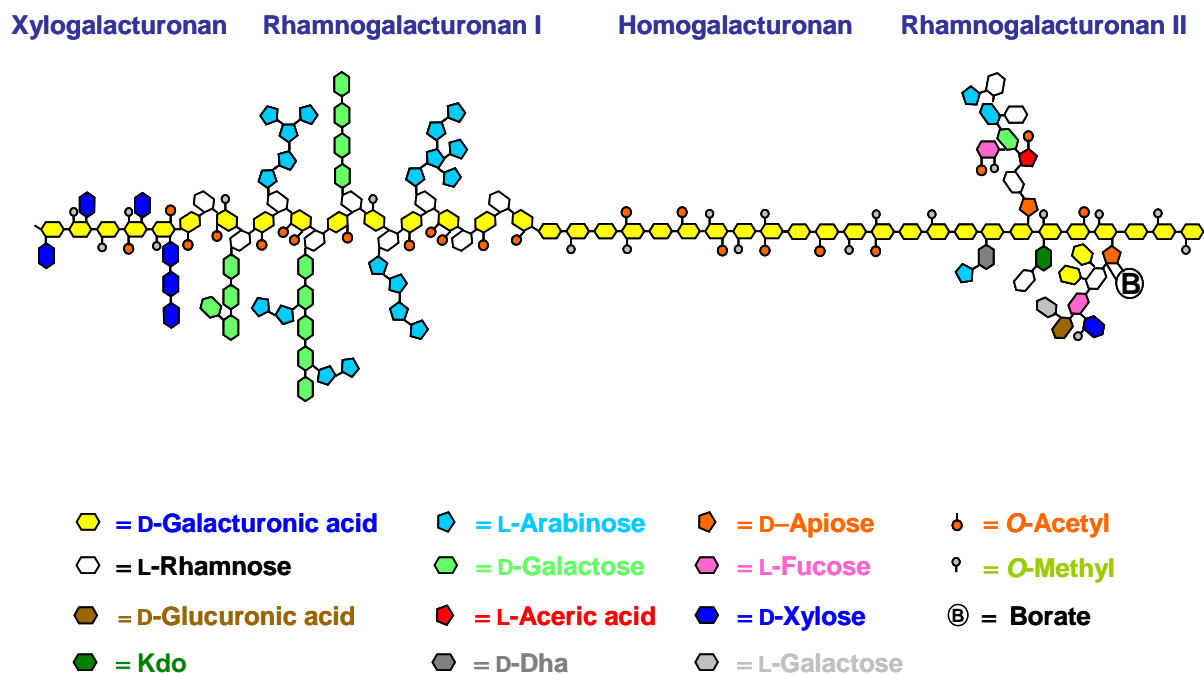**Xylogalacturonan** **Rhamnogalacturonan I** **Homogalacturonan** **Rhamnogalacturonan II**

Figure 1. Schematic structure of pectin. The figure illustrates the four different domains of pectin that are found ubiquitously. The relative abundance of the different types of pectin varies, but RGI and HGA are the major components whereas XGA and RGII are minor components.
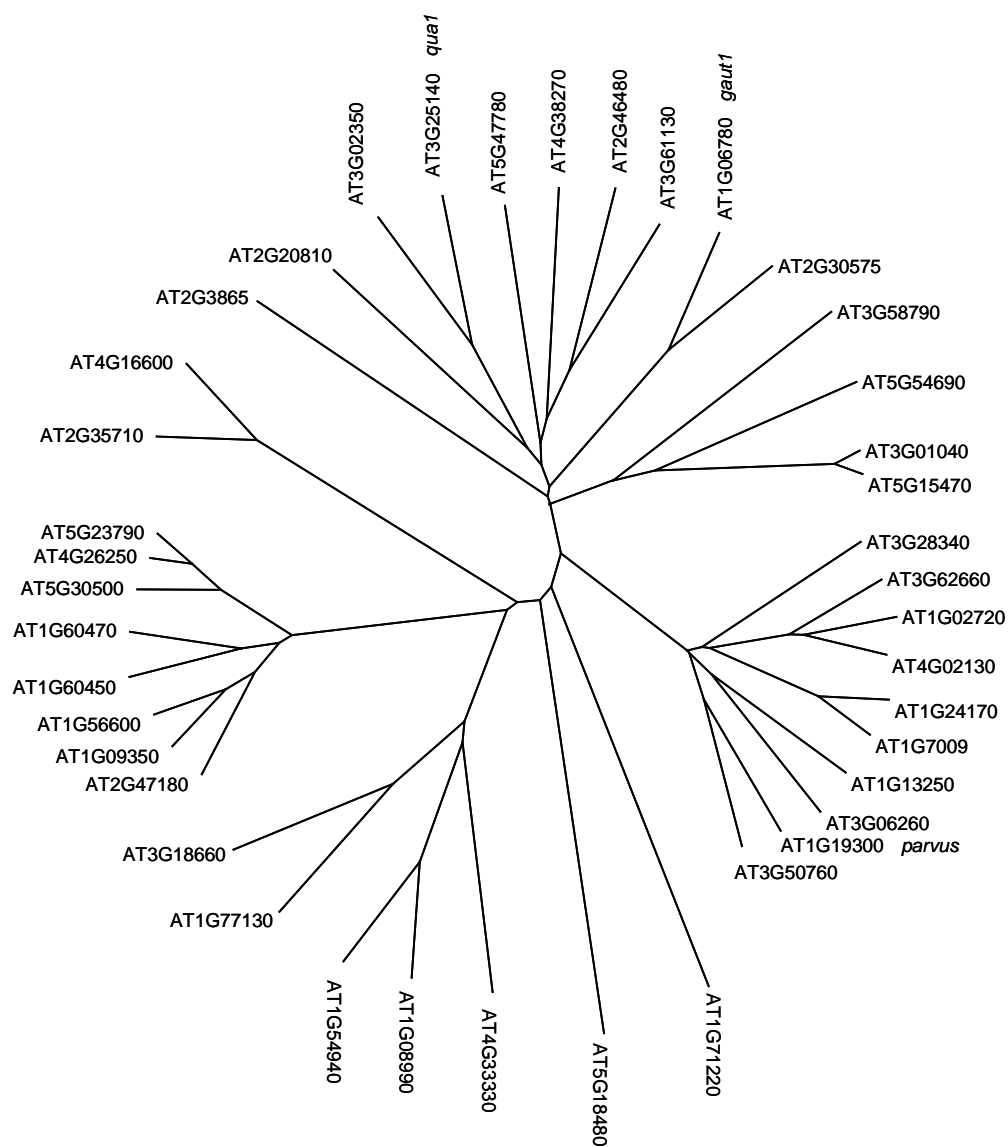
Figure 2. Phylogenetic tree of CAZy Family GT8 in Arabidopsis. The tree was constructed by aligning the sequences with Clustal X and drawing the tree in TreeView.
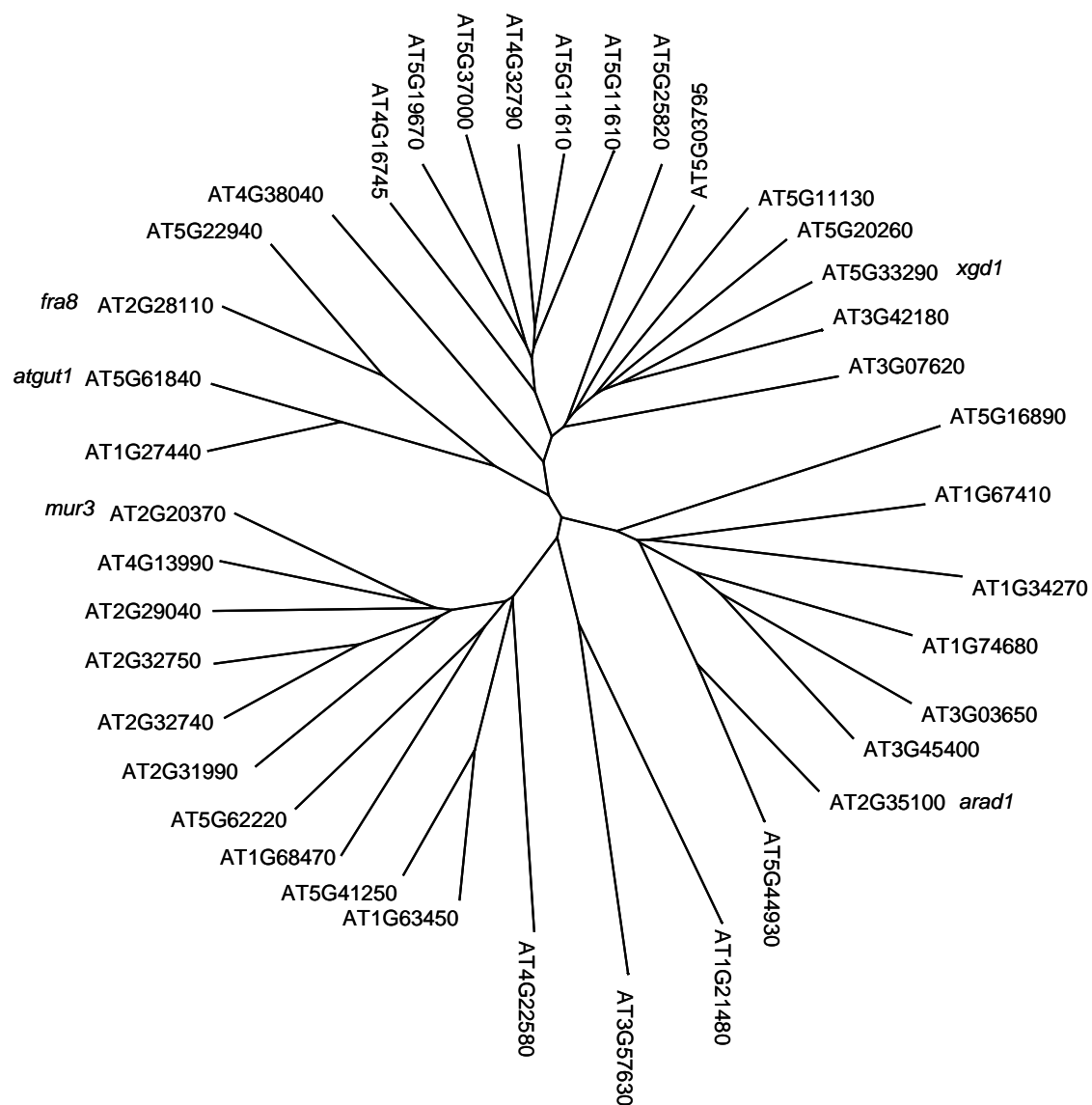
Figure 3. Phylogenetic tree of CAZy Family GT47 in Arabidopsis. The tree was constructed by aligning the sequences with Clustal X and drawing the tree in TreeView.

Due to restrictions from the publishers of the articles in chapter 3-7, these chapters are not available in this PDF. The articles can be found in:

Chapter 3:
Øbro, J., Harholt, J., Scheller, H. V., & Orfila, C. (2004). Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures. *Phytochemistry, 65*, 1429-1438.
DOI: 10.1016/j.phytochem.2004.05.002

Chapter 4:
Iain, W. M. (2004). Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *The Plant Journal, 40,* 260-275.
DOI: 10.1111/j.1365-313X.2004.02208.x

Chapter 5:
Sunil Kumar Singh, C. E. J. H. H. V. S. A. M. (2005). Cell adhesion in *Arabidopsis thaliana* is mediated by ECTOPICALLY PARTING CELLS 1 – a glycosyltransferase (GT64) related to the animal exostosins. *The Plant Journal, 43,* 384-397.
DOI: 10.1111/j.1365-313X.2005.02455.x

Chapter 6:
Orfila, C., Sørensen, S., Harholt, J., Geshi, N., Crombie, H., Truong, H. N. et al. (2005). QUASIMODO1 is expressed in vascular tissue of Arabidopsis thaliana inflorescence stems, and affects homogalacturonan and xylan biosynthesis. *Planta, 222,* 613-622.
 DOI: 10.1007/s00425-005-0008-z

Chapter 7:
Harholt, J., Jensen, J. K., Sorensen, S. O., Orfila, C., Pauly, M., & Scheller, H. V. (2006). ARABINAN DEFICIENT 1 Is a Putative Arabinosyltransferase Involved in Biosynthesis of Pectic Arabinan in Arabidopsis. *Plant Physiology, 140,* 49-58.
DOI: 10.1104/pp.105.072744

Chapter 3

The work leading to identification of a novel arabinogalactan structure in potato RG-I is presented. Furthermore is a method for rapid analysis of the major cell wall related sugars, based on HPEAC-PAD presented.

Chapter 4

The herbicide isoxaben disrupts the synthesis of cellulose. Plants response to isoxaben treatment by increasing the biosynthesis of hemicellulose and pectin. Microarray expression analysis was used for comparing isoxaben habituated suspension cells with wildtype and thereby increase our understanding of the biosynthesis of hemicellulose and pectin.

Chapter 5

Knock out of the gene *ECTOPICALLY PARTING CELLS 1*, a glycosyltransferase from CAZy family 64, results in plants with a dramatically reduced growth habit, defects in vascular formation and reduced cell-cell adhesion properties in hypocotyl and cotyledon tissues. No apparent changes in the cell wall sugar composition was observed.

Chapter 6

This chapter describes a thorough biochemical characterisation of *qua1*. *In vivo* measurements of cell wall biosynthetic activities were carried out possibly linking homogalacturonan and xylan biosynthesis.

Chapter 7

ARAD1 was identified as a putative arabinan arabinosyl transferase based on biochemical characterization of *arad1* plants. To our knowledge, the identification of other L-arabinosyltransferases has not been published.

Chapter 8

Xylogalacturonan was identified and characterized in vegetative tissue of *Arabidopsis thaliana*.

XGA exists in cell walls from various tissues of *Arabidopsis thaliana*

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Abstract

Evidence is presented for the presence of xylogalacturonan (XGA) in *Arabidopsis thaliana*. This evidence was obtained by extraction of pectin from the seeds, root, stem, young leaves and mature leaves of *A. thaliana*, followed by treatment of these pectin extracts with xylogalacturonan hydrolase (XGH). Upon enzymatic treatment, XGA oligosaccharides were primarily produced from pectin extracts obtained from the young and mature leaves and to a lesser extent from those originating from the stem of *A. thaliana*. The oligosaccharide GalA$_3$Xyl was predominantly formed from these pectin extracts. No XGA oligosaccharides were detected in digests of pectin extracts from the seeds and roots.

There was less variety of XGA oligosaccharides obtained from xylogalacturonan present in pectins from *A. thaliana* compared to the variety of oligosaccharides obtained from a xylogalacturonan derived from Gum Tragacanth. This indicates a more uniform distribution of xylose in XGA from *A. thaliana*. The predominant production of GalA$_3$Xyl, as well as the release of linear GalA oligosaccharides pointed to a lower degree of xylose substitution in XGA from *A. thaliana* than in XGA from apple and potato.

The estimated amount of XGA accounted for approximately 2.5 %, 7 % and 6 % (w/w) of the total carbohydrate in the pectin fraction of the stem, young leaves and mature leaves respectively.

Keywords: *Arabidopsis thaliana*; Pectin; Endo-xylogalacturonan hydrolase; Xylogalacturonan

Abbreviations used: MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; HPAEC, high performance anion exchange chromatography; PAD, pulsed amperometric detection; XGH, Xylogalacturonan hydrolase, XGA, xylogalacturonan; CWM, cell wall material; CWM-s, saponified cell wall material; RG-I, rhamnogalacturonan I; AIR, alcohol insoluble residue; AUA, Anhydro-uronic acid

1. Introduction

Primary cell walls are the major textural components of plant-derived foods. The most important polysaccharides that account for 90 to 100% of the structural polymers of these cell walls are cellulose, hemicellulose and pectin (Albersheim *et al.*, 1996).

Cellulose is comprised of β-(1→ 4)-linked D-glucan while hemicelluloses primarily consists of xyloglucan and arabinoxylan (Albersheim *et al.*, 1996). Pectin is a heteropolysaccharide, which contains α-(1→ 4)-linked D-galacturonic acid chains (also known as the smooth regions of pectins) and the branched polysaccharides rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan (referred to as the "hairy" regions)(Benen *et al.*, 2002; Schols and Voragen, 1996; Vincken *et al.*, 2003).

Cellulose exists in plant cell walls as microfibrils, which are long semicrystalline glucan chains (Gibeaut and Carpita, 1994). These microfibrils are cross-linked by hemicelluloses, such as xyloglucans that are believed to be a major factor in controlling the rate of cell-wall expansion (Gibeaut and Carpita, 1994; O'Neill and York, 2003; Zablackis *et al.*, 1995). The load-bearing cellulose-hemicellulose network is embedded in a matrix of pectic polysaccharides, which form a hydrated and crosslinked three-dimensional network (Gibeaut and Carpita, 1994; Knox, 2002).

Xylogalacturonan (XGA) is an α-(1→ 4)-linked D-galacturonic acid chain, which is substituted with β-D-Xylose at the O-3 position. It is suggested that this biopolymer is a side chain of RG-I (rhamnogalacturonan I) in the "hairy" regions of pectin (Vincken *et al.*, 2003).

The presence of XGA in plants has been reported in storage tissues of reproductive organs such as in cell walls of peas, soybeans, watermelons, apples, pears, onions, potato's, pine pollen, and cotton (Albersheim *et al.*, 1996; Le Goff *et al.*, 2001; Nakamura *et al.*, 2002; Schols *et al.*, 1995; Thibault and Ralet, 2001; Vincken *et al.*, 2003; Voragen *et al.*, 2001; Zandleven *et al.*, 2006; Huisman *et al.*, 1999). Its presence has also been reported in exudates from trees, such as Gum Tragacanth from the *Astralagus species* (Aspinall and Baillie, 1963).

Previous studies demonstrated that leaf primary cell walls of *A. thaliana* contain primarily homogalacturonan, RG-I, RG-II, xylan, xyloglucan and cellulose (Zablackis *et*

al., 1995). These polymeric structures were also suggested to be present in cell walls of the stem of this plant species (Gardner *et al.*, 2002). Additionally, mannan polysaccharides have also been reported in the cell walls of leaves and stem of *A. thaliana* (Handford *et al.*, 2003).

So far, the presence of other polysaccharides, such as XGA, has not been found in *A. thaliana* (Zablackis *et al.*, 1995), although it has been suggested that this polymer exists in root caps of this plant species. This was based on immunocytochemical analysis using an LM8 antibody that was raised against pea XGA (Willats, 2004). Also Gardner *et al.* (2002) mentioned XGA as a probable xylose-containing polysaccharide in the stem of *A. thaliana*; however this was only based on the sugar composition of the hydrolysis products from the alcohol insoluble residue (AIR) of the stems.

Recently, a gene (At5g33290) expected to encode a β-xylosyl transferase was identified from *A. thaliana*. Pectin isolated from a T-DNA insertion line having a T-DNA insertion in this particular gene was found to contain less xylose compared to the wild type (Sørensen, Jensen, Harholt, Scheller, in preparation). We therefore hypothesized that pectin from *A. thaliana* may contain regions of XGA. To investigate this, pectin was extracted from cell wall material, prepared from the seeds, roots, young leaves and mature leaves of *A. thaliana*. The pectin fractions were analysed for their sugar composition and subsequently treated with xylogalacturonan hydrolase (XGH) to determine the presence of XGA. Based on the obtained XGA oligosaccharides, the amount of degraded XGA was estimated and some structural characteristics of XGA were investigated.

Results

2.1 Analysis of CWM

CWM was prepared from the seeds, roots, stem, young leaves and mature leaves of *Arabidopsis thaliana*. In line with other reports (Zablackis *et al.*, 1995), polysaccharides possibly lost during some of the preparation steps (i.e. starch and protein removal) were not considered as cell wall components.

These CWM was saponified to ensure full removal of methyl- and acetyl esters, which could interfere with subsequent enzymatic treatment. The sugar composition of the saponified CWM (CWM-s) samples is shown in Table I. All the samples contained high amounts of glucose and significant amounts of xylose, which suggests the presence of cellulose, xylan and xyloglucan. The presence of these polysaccharides was also indicated in cell walls of *A. thaliana* leaves, as shown by Zablackis *et al.*, 1995.

Besides glucose and xylose, also rhamnose, arabinose, galactose and in particular high amounts of galacturonic acid were detected, which implies that these samples also contain pectin. The presence of the major sugars, glucose and galacturonic acid has also been demonstrated previously for *A. thaliana* stems (Gardner *et al.*, 2002) and *A. thaliana* leaves (Zablackis *et al.*, 1995) although different pectin extraction techniques were used.

2.2 Analysis of pectin extracts

To investigate the presence of xylogalacturonan (XGA) in the cell walls of seeds, roots, stem, young leaves and mature leaves of *A. thaliana* it was required to extract the pectin from CWM-s of these plant organs first. The solubilized material, as extracted from these CWM-s, which contain the pectic polysaccharides will be referred to as "pectin fractions". Based on the yields and the sugar compositions it was estimated that the amount of carbohydrate in the pectin fractions accounted for approximately 8 %, 16 %, 17 %, 21 % and 20 % (w/w) of the carbohydrate content of the CWM-s from the seeds, roots, stem, young leaves, and mature leaves respectively.

As expected for the method used (Voragen *et al.*, 1983), the sugar composition of all the pectin fractions shows that galacturonic acid is the major sugar (Table II). Compared to the sugar composition of the CWM-s samples, the amount of glucose is significantly reduced in the corresponding pectin fractions, which illustrates that pectin was selectively extracted.

Arabinose and galactose are the major neutral sugars in pectin fractions from the root, stem and leaves. This was also found in pectin extracts from several fruit and vegetables (Voragen *et al.*, 1983). The arabinose and galactose contents suggest that RG-I with side chains of arabinan and/ or arabinogalactan exists in these pectin fractions.

A relatively low content of galactose was observed in the pectin fraction from the seeds. On the other hand a high amount of rhamnose was found in this pectin fraction. This has also been reported before for the ammonium oxalate extracted mucilage from the seeds of *A. thaliana* (Usadel *et al.*, 2004). This implies that RG-I is a major component in pectin from the seeds of *A. thaliana*.

In general, the presence of both galacturonic acid and xylose in all pectin fractions (Table II) indicates that these fractions contain XGA. To investigate this, these fractions were treated with XGH in order to study the release of XGA oligosaccharides (see section 3.3). The major sugars in the pellet fractions of the different CWM-s extracts from roots, stem, young leaves and mature leaves were glucose and xylose (Table III), which points to the potential presence of cellulose, xylan, and xyloglucan. Besides glucose and xylose, these pellet fractions also contain uronic acid. Apart from glucose, the pellet fraction of the seeds is particularly rich in this monosaccharide. Based on the sugar composition of the pellet fractions it is assumed that uronic acid occurs as glucuronic acid as well as galacturonic acid in these fractions. This is based on the results shown in Table III, in which the presence of arabinose, xylose and uronic acid suggests that these fractions contain the hemicellulosic polysaccharide glucuronoarabinoxylan. A similar conclusion was made by Zablackis *et al.*, 1995. Besides this, also pectin related sugars were found in these pellet fractions, which point at the presence of galacturonic acid.

The pellet fraction from the seeds has a relatively higher content of rhamnose and galactose, compared to other pellet fractions, which indicates that this fraction contains a relatively high amount of rhamnogalacturonan. This latter pellet fraction also had a 3

times higher galactose content than its corresponding pectin fraction, while the rhamnose content in this pellet fraction was approximately 4 times lower. Also an equal proportion of arabinose was found in the pellet, compared to its corresponding pectin fraction. These results indicate that, although a major part of the pectin was extracted, the pellet fraction of seeds still contains rhamnogalacturonan I with side chains of arabinans and/ or arabinogalactans.

2.3 Evidence for XGA in *A. thaliana*

The presence of XGA in the pectin fractions was investigated using the enzyme XGH as analytical tool. The pectin fractions were treated with XGH for 16 h, prior to analysis by HPAEC and MALDI-TOF MS.

As demonstrated by HPAEC (Fig. 1), ten different oligosaccharides were identified in the mature leaf digest. The main product was $GalA_3Xyl$, while significant amounts of GalA, GalAXyl, $GalA_2Xyl$, $GalA_4$, $GalA_5$ and $GalA_6$ and minor quantities of $GalA_2$, $GalA_2Xyl_2$ and $GalA_2Xyl'$ were observed. The accentuated oligosaccharide $GalA_2Xyl'$ is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in $GalA_2Xyl$ (Zandleven *et al.*, 2005).

Comparable results were acquired for young leaves (Fig. 2) and stem (results not shown), although a significantly lower level of the oligosaccharides $GalA_4$, $GalA_5$ and $GalA_6$ was released from pectin from the young leaves.

The formation of a series of oligosaccharides, as presented in Figures 1 and 2, was confirmed by MALDI-TOF MS (results not shown). From this it can be concluded that XGA is present in the stem, young leaves and mature leaves of *A. thaliana*. Compared to the release of XGA oligosaccharides from XGA-29 (Zandleven *et al.*, 2005), only a low variety XGA oligosaccharides was observed for the XGH treated pectins from the stem and the leaves. This implies that the distribution of xylose side chains over the backbone in XGA from *A. thaliana* is relatively more uniform than the xylose distribution in XGA from Gum Tragacanth. The degree of xylose substitution in XGA from pectin from the stem and the leaves is lower than that of XGA from apple pectin and potato pectin (Zandleven *et al.*, 2006). While XGH mainly released the disaccharide GalAXyl from

these latter two sources, a predominant production of GalA$_3$Xyl was seen for the *A. thaliana* pectins. Also the relative amount of linear oligosaccharides in relation to branched GalA oligosaccharides was higher in the *A. thaliana* pectins than in pectins from apple and potato (Zandleven *et al.*, 2006).

As obtained by HPAEC and MALDI-TOF MS analysis (results not shown), the presence of XGA could neither be demonstrated in pectin from the seeds nor from the roots of *A. thaliana*, although it has been indicated that XGA exists in root caps of *A. thaliana* (Willats *et al.*, 2004). It is possible that the concentration of XGA is too low in these pectin samples to be detected by our methods. If this is the case, it is possible that xylose in these samples originates from xylose containing polysaccharides other than XGA. Alternatively, it is also possible that XGH-resistant XGA exists in these pectin fractions. The occurrence of XGH-resistant XGA has also been observed before for pea XGA (Beldman *et al.*, 2003).

Although the pellet fractions from the different plant organs contained some residual pectin material, no XGA oligosaccharides were detected by HPAEC and MALDI-TOF MS analysis (results not shown) of these XGH-treated pellet fractions. This is probably due to similar reasons as described above.

The total amount of liberated XGA from the pectin fractions of the stem, young leaves and mature leaves was estimated. For this, the amount of each XGA oligosaccharide in the different pectin digests was quantified and the total amount determined by summation of these values. The enzyme XGH was also able to release linear GalA oligosaccharides from these pectin fractions. Based on the fact that XGH degrades neither polygalacturonic acid (Beldman *et al.*, 2003) nor galacturonic acid oligosaccharides (Zandleven *et al.*, 2005), it is concluded that these linear GalA oligosaccharides are also products from XGA and were therefore included in the calculations. These linear GalA oligosaccharides can be produced by XGH from unsubstituted regions of XGA by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA.

Approximately 2.5 %, 7 % and 6 % (w/w) of XGA related products could be liberated from the total carbohydrate present in the pectin fraction from the stem, young leaves and mature leaves respectively. Using these results, a calculated guess was made for the degree of XGA degradation in these pectin samples. To do this, first the theoretical

amount of XGA in these pectin fractions was calculated. For this it was assumed that the GalA:Xyl ratio, on average, is 3:1, based on the predominant production of GalA$_3$Xyl. As a similar type of XGA oligosaccharide production was observed for all these pectin fractions, this GalA:Xyl ratio was used to calculate the theoretical amount of XGA for each fraction.

From the amount of XGA oligosaccharides estimated from HPAEC analysis and the theoretical amount of XGA, it was calculated that approximately 40 % of XGA has been degraded in pectin from the young leaves and mature leaves, while only 7 % of XGA was degraded in pectin from the stem. However, as these calculations were based on the assumption that xylose is exclusively present in XGA in these pectin fractions, it is likely that the theoretical content of XGA in these fractions is overestimated. This implies an underestimation in the percentages of XGA degradation mentioned above. Although pectin was selectively extracted from the leaves and the stem of *A. thaliana*, it can not be excluded that xylose containing polysaccharides other than XGA, such as xylan and xyloglucan, exist in these pectin samples. This may in particular be the case for the pectin fraction of the stem, which contains a higher amount of xylose compared to the pectin fractions from the leaves.

Indeed, only the degradable part of XGA is taken into account for the calculation of the degree of XGA degradation for these fractions. It cannot be ruled out that XGH resistant parts of XGA are also present in these pectin samples, which negatively affect the degree of XGA degradation.

The results from this study clearly demonstrate the presence of XGA in the stem, young leaves and mature leaves of *A. thaliana*, although a relative lower amount of this polymer was detected in the stem compared to the leaves.

To our knowledge this is the first time that the occurrence of XGA has been demonstrated in plant material other than storage or reproductive tissues and root caps. The outcome of this study may initiate investigations on the presence of XGA in other plant varieties, as well as the relationship between XGA structure and its function in different plant tissues.

3 Methods

3.1. Plant Material

Arabidopsis thaliana (L) Heyn. Ecotype Col-0 was used for all experiments.

Plants were grown in peat at an 8 h photoperiod at 100-120 µmol photons m^{-2} s^{-1}, 20 ^{o}C, 70 % relative humidity and watered using tap water when necessary.

Plant material (green tissue) was harvested as young leaves (small rosettes 10 to 12 leaf stage), mature leaves (12 weeks), stems (including flowers and silliques) and seeds. Roots were obtained from plants grown hydroponically according to Husted *et al*. (2002).

3.2 Preparation of cell wall material (CWM)

Cell wall material was prepared as follows: alcohol insoluble residue (AIR) was prepared as described (Fry, 1988) with adaptations. Tissue of interest was ground in liquid nitrogen with a mortar and pestle and boiled in 96% ethanol for 30 min. The supernatant was removed after centrifugation at 10.000 g for 5 min. The pellet was washed with 70% ethanol with subsequent centrifugation until it appeared free of chlorophyll. A final wash with 100% acetone was performed and the pellet was dried under vacuum.

Alcohol insoluble residue (AIR) was treated with enzymes for the removal of starch. For this, AIR was suspended in 10 volumes of a solution that had been pre-heated to 95°C containing 10 mM potassium phosphate buffer (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3. Starch was allowed to gelatinize for 30 s before addition of thermostable α-amylase (Megazyme, Bray, Ireland) to a final concentration of 1 U/ml. The suspension was incubated at 85°C for 15 min. After the incubation the sample was cooled to 25°C and amyloglucosidase and pullulanase (both from Megazyme) were added to a final concentration of 1 U/ml. The suspension was incubated for 16 h at 25 °C, with continuous shaking at 500 rpm. The suspension was centrifuged for 10 min at 6000 g. The pellet was washed with 50 ml of a solution containing 10 mM potassium phosphate (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3, centrifuged again at 6000 g for 10 min, and

finally dried under vacuum. The de-starched cell wall material was extracted with phenol:acetic acid:water (2:1:1, v/v/v) for 3 h (1:10 (w/v) ratio between AIR and phenol:acetic acid:water 2:1:1) at room temperature followed by centrifugation at 6000 g for 5 min. The pellet (CWM) was washed three times with water to remove phenol and extracted proteins and finally dried under vacuum.

3.3. Saponification of CWM

Pectin, as present in CWM from seeds, root, stem, young leaves and mature leaves was saponified in 0.1 M sodium hydroxide for 24 h at 4 ºC and subsequently neutralized with 0.1 M acetic acid. After neutralization, samples were dialyzed overnight against MilliQ water and freeze-dried until further use. Saponified CWM is referred to as CWM-s.

3.4. Pectin extraction from CWM-s

Pectin was extracted from saponified CWM (CWM-s) from the different plant organs according to the method of Voragen *et al*. (1983). For this extraction a cold solution of 5 mM EDTA in 50 mM NaOH (4 °C) was used.

The obtained pectin and pellet fractions from each type of CWM-s were dialyzed overnight against distilled water. Aliquots of these fractions were freeze-dried prior to analysis of their sugar compositions (see section 2.5). The total concentration of soluble polysaccharides in the pectin fractions as well as the total concentration of the residue in the pellet fractions was calculated using the yield of dry material from the freeze dried aliquots of these fractions.

The remainder of the pectin fractions as well as the pellet fractions were dialyzed against 50 mM NaOAc (pH 3.5) prior to treatment by XGH, see section 2.7.

3.5. Neutral sugar composition and uronic acid content

CWM-s, as well as the corresponding pectin and pellet fractions, were analyzed for their neutral sugar compositions using gas chromatography, after derivatization to alditol acetates (Englyst and Cummings, 1984). Inositol was used as internal standard. The samples were hydrolyzed in 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a treatment in 1 M H_2SO_4 for 3 h at 100 °C. The uronic acid content of the samples was determined

by an automated m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault and Robin, 1975).

3.6 Substrates and enzymes

Xylogalacturonan (XGA-29) was prepared from Gum Tragacanth by treatment with alkali and trifluoro acetic acid (TFA) as described (Beldman *et al.*, 2003). This XGA had a Xyl:GalA ratio of 0.29.

A set of XGA oligosaccharides with known structures and with different GalA/ Xyl ratios were obtained as described (Zandleven *et al.*, 2005).

The enzyme xylogalacturonan hydrolase (XGH) from *Aspergillus tubingensis* was cloned (Van der Vlugt-Bergmans *et al.*, 2000) and expressed in the *A. niger* "PlugBug" (DSM Food Specialities, Delft, the Netherlands). This enzyme was purified as described (Beldman *et al.*, 2003) and had a specific activity of 150 U/mg.

3.7. Enzyme incubations

One ml from the dialyzed pectin or pellet fraction (ranging in concentration between 1 to 6 mg/ml) from the different plant organs was incubated with XGH for 20 h at 30 ºC. The final enzyme concentration was 0.35 µg/ml, which should be able to degrade all XGA possibly present in these samples during the incubation period. Subsequently, the enzyme was inactivated by heating the reaction mixtures for 10 min at 100 ºC. The XGH treated samples were analyzed by HPAEC using a set of known XGA-oligosaccharides for identification (Zandleven *et al.*, 2005). GalA, $GalA_2$, and $GalA_3$, (all 10 mM) were taken as standards to calculate the concentration of the corresponding oligosaccharides, as well as of the xylogalacturonan oligosaccharides. For the calculation of the XGA oligosaccharides it was assumed that xylose substitution has no significant effect on the response factor of galacturonan oligosaccharides (Sakamoto *et al.*, 2002). The standard $GalA_3$ was also used to calculate concentrations of oligosaccharides with a degree of polymerization (DP) of 4 and higher, based on the experience that the differences in response factors for these larger oligosaccharides are relatively small.

3.8 MALDI-TOF mass spectrometry

XGH-treated pectin samples were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per ml digest. The desalted digests were mixed with a matrix solution (1 μl of sample in 1 μl of matrix) and applied on a MALDI sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5- dihydroxybenzoic acid in a 1-ml mixture of acetonitrile:water (300 μl: 700 μl). MALDI-TOF MS analysis was performed using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions, which were accelerated to a kinetic energy of 12 kV, after a delayed extraction time of 200 ns. The ions were detected in the reflector mode. External calibration of the mass spectrometer was performed using a mixture of maltodextrines (mass range 365 to 2309).

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Legends to the figures

Figure 1. HPAEC of pectin from mature leaves of *A. thaliana*, respectively untreated (bold line) and treated for 20 h with XGH (thin line). The accentuated oligosaccharide GalA$_2$Xyl**'** is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in GalA$_2$Xyl

Figure 2. HPAEC of pectin from young leaves of *A. thaliana*, respectively untreated (bold line) and treated for 20 h with XGH (thin line). The accentuated oligosaccharide GalA$_2$Xyl**'** is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in GalA$_2$Xyl

Table I. Sugar composition (mol%) of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

	(mol %)							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA
Mature leaves	3	1	8	9	5	7	33	35
Young leaves	2	1	7	8	6	8	37	33
Stem	1	0	6	20	7	5	43	19
Root	2	1	18	14	4	7	35	20
Seeds	8	1	18	8	3	11	27	25

Table II. Sugar composition (mol%) of pectin extracts of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

Pectin extracts	(mol %)							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA
Mature leaves	3	1	9	7	5	9	5	64
Young leaves	4	0	9	6	5	9	5	64
Stem	3	0	16	13	2	10	4	52
Root	2	1	10	6	5	10	3	63
Seeds	15	0	17	5	3	4	7	70

Table III. Sugar composition (mol%) of pellet fractions after pectin extraction of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

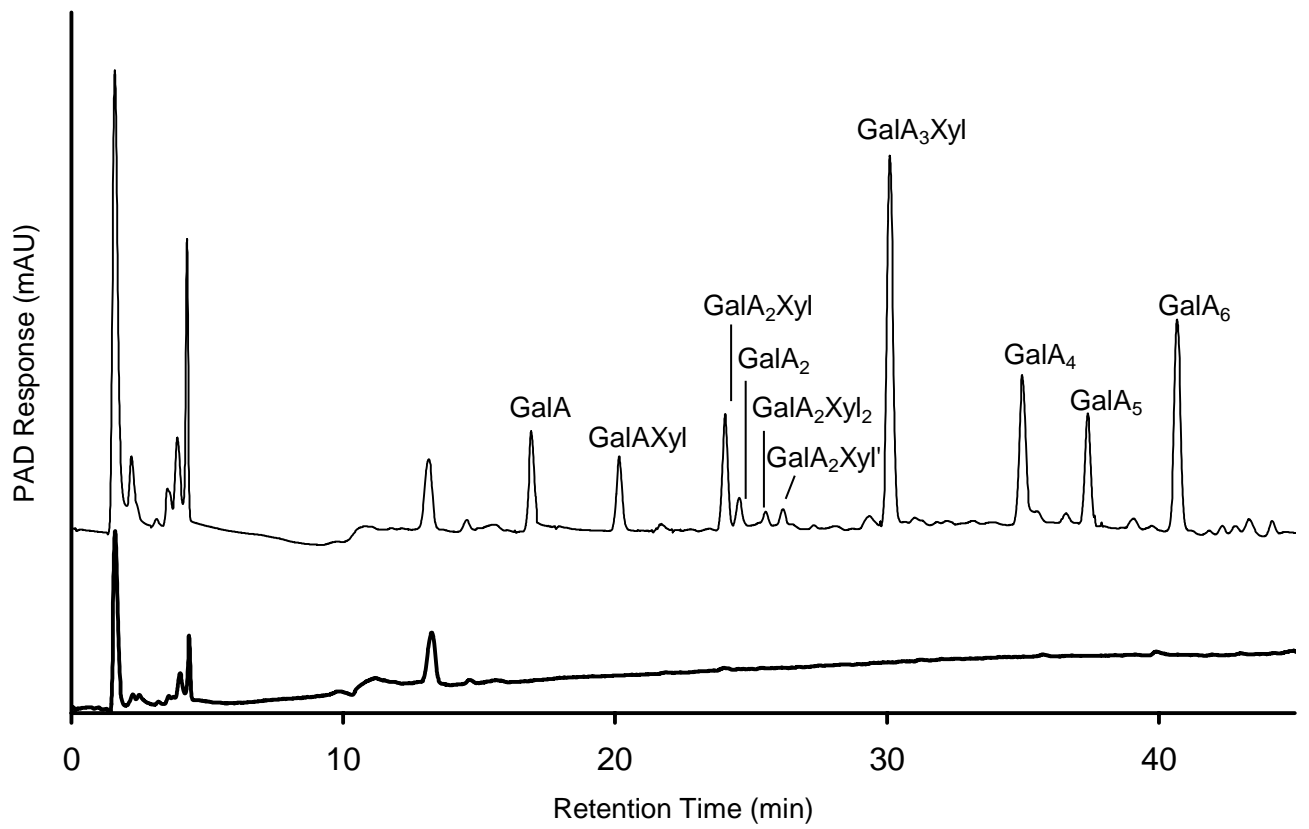| Pellets	(mol %)							
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA
Mature leaves	2	1	9	12	4	6	48	19
Young leaves	1	0	8	13	5	7	51	17
Stem	2	1	3	23	4	3	57	9
Root	2	2	18	14	3	7	44	10
Seeds	4	1	19	7	3	12	28	27

Figure 1

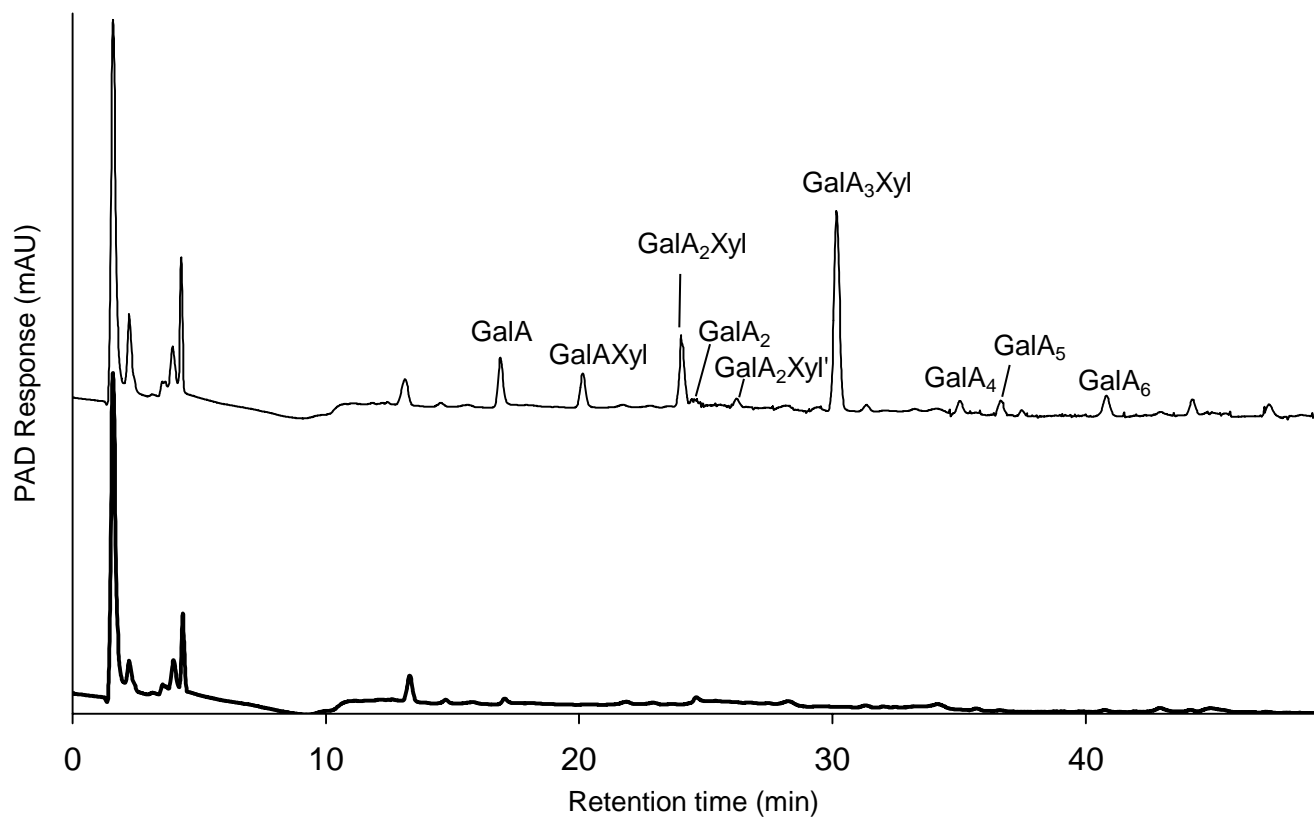Figure 2

Chapter 9

XGD1 was identified as a putative xylogalacturonan xylosyltransferase. The putative function was suggested as a result of the biochemical characterization of *xgd1-1* plants. Additionally it was suggested that xylogalacturonan exist in Arabidopsis as at least two distinct structures.

XYLOGALACTURONAN DEFICIENT I is a Putative Xylosyltransferase Involved in Biosynthesis of Xylogalacturonan in Arabidopsis

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MATERIALS AND METHODS

Plant Material

All Arabidopsis (Arabidopsis thaliana L. Heyn.) lines used in this work are derived from ecotype Col-0. Seed of T-DNA insertion line SAIL 1175_H04 (*xgd1-1*; insert placed approximately 300 bp upstream of stop codon in the last exon) and the corresponding background line (*qrt*) were obtained from Syngenta. T-DNA insertion line SALK_087620 (*xgd1-2*; insertion placed 90 bp upstream of stop codon in last exon) was obtained from the Salk institute (Alonso *et al.*, 2003; Fig.1).

Plants were grown in peat at an 8-h photoperiod at 100 to 120 μmol photons m^{-2} s^{-1}, 20°C, 70% relative humidity and watered using tap water as necessary. Fertilizer was not used. Young leaves were harvested at the 10 to 12 leaf stage and adult leaves after approximately 12 weeks of growth. To initiate bolting and synchronize stem growth, plants were shifted to a 16-h photoperiod at 100 to 120 μmol photons m^{-2} s^{-1} after 8 weeks growth in the 8-h photoperiod. Roots for qRT-PCR were obtained from plants grown hydroponically (Husted *et al.*, 2002). Roots for immunolabelling were obtained from 5 day old seedlings grown vertically on Murashige and Skoog medium (pH 5.7) containing 1% sucrose and 0.8% bacto-agar (Difco).

Bioinformatics

Protein targeting was predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/ (Emanuelsson *et al.*, 2000)), transmembrane helix prediction was performed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/ (Sonnhammer *et al.*, 1998)) and *N*-glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). *In silico* expression analysis and response viewer was performed using the GENEVESTIGATOR data mining tool, which is based on publicly available microarray experiments (Zimmerman *et al.*, 2004). Reannotation of At3g42180, At5g11130, At5g20260 and At5g25310 was done by investigating the upstream sequences of the respective genes as far away as 3 kb.

First the sequence imidiatly upstream the startcodon was translated in frame with the start codon. All three genes revealed highly similar sequences for the first five amino acids and importantly conserved among the rest of the genes in the group. Alignments were performed using ClustalX as in Harholt *et al.*, 2006.

Identification of homozygous plants.

Genomic DNA was prepared as described in Edwards *et al.*, (1991). *xgd1-1* homozygous plants were identified by PCR using primers suggested by Syngenta (gene specific primers 5'-GGGTTTAGCTGGGACGATTCAA-3' and 5'-CCGACCTAACGTGGACGTAT-3', insert specific primer 5'-TAGCATCTGAATGTCATAACCAAT-3'). For *xgd1-2* the T-DNA insert was annotated to be located in the last exon of *XGD1* approximately 500 base pairs upstream the stop codon according to the SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress, Alonso *et al.*, 2003). However, when potential T-DNA mutant plants were screened by PCR using primers suggested by the Salk Institute (forward gene specific primer 5'-TCAAGACGAATGAGTCGGAG-3' and reverse gene specific primer 5'-CCAAATTTTGTTTGGCTTGAA-3', insert specific primer, LBa1, 5'-TGGTTCACGTAGTGGGCCATCG-3'; Fig.1) an unexpected large insert product was obtained using the forward gene specific primer for *XGD1-2* in combination with the T-DNA insert specific primer LBa1. This indicated that the true location of T-DNA insertion was in fact much further downstream in the *XGD1* gene than initially identified. This hypothesis was verified by sequencing of the PCR product generated by the forward primer and LBa1 using sequencing primer 5'-CATTACCATTCAGTGATGTGC-3'. From this sequence (see Supplementary Fig.1) the protein sequence of the XGD1-2 protein could be deduced.

Gene expression analysis

Total RNA was isolateded from roots, young leaves, adult leaves, stems and flowers using RNeasy Plant Mini Kit (Qiagen, Germany).

cDNA was prepared from 1 µg total RNA using iScript cDNA synthesis Kit (Bio-Rad, Denmark) in a total volume of 25 µl. One µl was used for Real Time RT-PCR amplification of *XGD1* with gene specific primers (5'-CCATGACTGGCACCAGACGT-3' and 5'-TATCCTAGAAACAGGGATTTGTATGG-3') using ICycler Instrument (Bio-Rad, Denmark) with the iQ SYBR Green Supermix kit for PCR (Bio-Rad, Denmark) according to the manufacturer's instructions. The reactions were incubated at 96°C for 3 min. to activate the hot start recombinant Taq DNA polymerase, followed by 50 cycles of 1 min. at 96°C, 1 min. at 60°C and 1 min. at 72°C. The specificity of the PCR amplification was checked with a heat dissociation protocol (from 65 to 95°C) following the final cycle of the PCR. The results obtained for the different samples analysed were standardized to the polyubiquitin 10 RT-PCR product level using the primers 5'-GGCCTTGTATAATCCCTGATGAATAAG-3' and 5'-AAAGAGATAACAGGAACGGAAACATAGT-3'.

Preparation and chemical analysis of cell wall material

Cell wall material and subsequent RG I extraction was prepared from leaves as alcohol insoluble residue as previously described (Harholt *et al.*, 2006). Monosaccharide composition analysis was performed on alcohol insoluble residue and RG I isolated from leaves as in Øbro *et al.*, (2004). Glycosidic linkage analysis was performed as previously described (Harholt *et al.*, 2006).

Immuno-dot blot analysis

CCRCM1 (FucXylGlc)

CMB2B-1 (AraXylan)

Extraction and analysis of xylogalacturonan by HPAEC and MALDI-TOF MS

Pectin was extracted from cell wall material isolated from adult leaves from *qrt* and *xgd1-1* plants as described by Zandleven *et al.*, In preparation.

The obtained pectin fractions were dialyzed overnight against distilled water and subsequently against 50 mM NaOAc (pH 3.5) prior to treatment with xylogalacturonan endohydrolase (XGH)(Beldman *et al.*, 2003). The degradation products were analysed by HPAEC and MALDI-TOF MS as previously described (Zandleven *et al.*, In preparation).

Immunolabeling and microscopy

Leaf material and siliques from four week old *xgd1-1* and *qrt* plants were placed in fixative (2% par formaldehyde, 2.5% gluaraldehyde, 0.1M Phosphate buffer pH7) and placed under vacuum for 30 minutes. The plant material was then left in fixative overnight at 4°C and washed twice in 0.1M Phosphate buffer pH7 for 30 min each time at 4°C. Samples were dehydrated in an ethanol series (30, 50, 70, 80, 90, 100%) for 20 minutes each and then transferred to LR White resin (Hard Grade Acrylic Resin; London Resin Company Ltd, Berkshire, UK) at 4 ^{o}C for 24 hrs with 2 changes to allow the resin to infiltrate the tissue. The plant material was then placed in gelatine capsules and filled with LR White and polymerized at 60°C for 24 hrs. Transverse sections of leaf and siliques material for both *xgd1-1* and *qrt* were cut at a thickness of 3μm with glass knives on a microtome (LKB Bromma 2218 Historange Microtome) and placed on diagnostic microscope slides (Erie Scientific Co., Portsmouth, UK) that were coated in vectorbond reagent (SP-1800; Vector Laboraties Inc., Burnlingame, CA, USA). Resin-embedded sections of siliques and leaves and 5 day old root tips from *xgd1-1* and *qrt* plants for surface labeling were blocked for 1hr in 5% milk powder in PBS and then incubated with 10-fold dilutions of LM8 epitope specific for xylogalacturonan. In addition leaf resin-embedded sections were incubated with 10-fold dilution of JIM5 epitope for partially methyl-esterified homogalacturonan. After washing the sections and surface labeled roots were incubated with 100-fold dilution of fluorescein isothiocyanate (FITC; Sigma) conjugated secondary antibody and leaf sections were counterstained for 5 min with Calcofluor White M2R fluorochrome (fluorescent brightener 28; Sigma; 20mM in phosphate buffer pH8) to detect the presence of cellulose.

After washing with PBS the sections were mounted in a glycerol/PBS based anti-fade solution (Citifluor AF1; Agar Scientific) and observed on an Epifluorescence microscope (Zeizz, Axioplan).

Transformation of Plants with *35S::XGD1* constructs

All constructs were generated on the basis of the *XGD1*-cDNA clone MPIZp2001I033Q2 (RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) and by the use of Phusion polymerase (Finnzymes). The *XGD1* coding region was amplified using forward primer 5'-GTCCG**GAGCTC**ATGGCTGCTCCAAGATC-3' (*Sac*I underlined) and reverse primer 5'-GACAT**GCATGC**CTATGTACCAAGCCTAAGATTAAG-3' (*Sph*I underlined). Alternatively, At5g33290 has been annotated to encode a soluble protein being 160 amino acids shorter from the N-terminal compared to the above annotation. This version of the gene was amplified using forward primer 5'-GTCCG**GAGCTC**ATGAATCGTTTTAAGGTGTG-3' together with the same reverse primer as above. The PCR products were cloned as *Sac*I/*Sph*I fragment under control of the cauliflower mosaic virus (CaMV) 35S promoter and terminator in pPS48 (Kay *et al.*, 1987), resulting in pJJ28 and pJJ30, respectively. A fusion protein was generated by adding a tag to the C-terminal end of the XGD1 protein. This construct was generated by inserting the c-myc-6xHis cassette from the pPICZαA plasmid (Invitrogen) into pPS48 as a *Sac*I/*Sph*I fragment. This fragment was amplified using the following primers : 5'-GTCCGGAGCTCGCGGAACAAAAACTCATCTCAGAAG-3' (*Sac*I underlined) and 5'-GACATGCATGCGCAAATGGCATTCTGACATCC-3' (*Sph*I underlined). The complete *XGD1* coding region without stop codon was inserted as a *Sac*I/*Sac*I fragment generated by forward primer above and the reverse primer 5'-GTCCGGAGCTCTGTACCAAGCCTAAGATTAAG-3' (*Sac*I underlined) resulting in pJJ29. Insert and vector-insert junctions were sequenced, and subsequently, the *Xba*I fragment of pJJ28, pJJ29 and pJJ30 containing the CaMV 35S promoter, insert and the CaMV 35S terminator was transferred to pPZP221 (Hajdukiewicz *et al.*, 1994).

qrt and *xgd1-1* plants were transformed with empty pPZP221 vector or with the vectors containing the different p35S::*XGD1*-versions by *Agrobacterium tumefaciens*-mediated transformation using the strain C58C1 pGV3850. Seeds were selected for 2 weeks on Murashige and Skoog medium containing 100 µg/mL gentamycin sulphate and subsequently transferred to soil and grown as described above. After 8 weeks on soil full grown rosette leaves were harvested and used to prepare total cell wall alcohol insoluble residue for determination of monosaccharide composition.

Fluorescent protein fusion and subcellular localization

The full length *XGD1* was amplified from the plasmid XXX using the following primers: NT01, 5´-GGCTTAAUATGGCTGCTCCAAGATCAAG-3´, and NT02, 5´-GGTTTAAUCCTGTACCAAGCCTAAGATTAAGTC-3´, in which the underlined oligonucleotides were engineered to provide USER cloning sites (unpublished). The fragments were inserted into the unique USER cassette in the plasmid pNB16YFP (I need to double check this name). This generates the plasmid pYS17 carring the C-terminal translational fusion of XGD and a yellow fluorescent protein (YFP). The previously described constructs containing the translational fusions STtmd-GFP (green fluorescent protein) and GFP-HDEL were used as the markers specific for Golgi apparatus and ER, respectively (Latijnhowers *et al.*, 2005). *Agrobacterium tumefaciens* C58C1 pGV3850 were transformed with each construct by electroporation and were selected for resistance against kanamycin (50µg/ml). For transformation of *Nicotiana benthamiana*, these Agrobacteria were grown over night in LB containing 10mM MES, and 20µM acetosyringone. The bacterial cells were harvested by centrifugation and resuspended in a buffer containing 10mM MES, 10mM $MgCl_2$, and 100µM acetosyringone (OD600nm=0.05) and allowed to stand at RT for 2-3 h. Leaves of the wild-type *N. benthamiana* (6 weeks old, grown in greenhouse at 28ºC/day and 18ºC/night with xx h light) were infiltrated with the bacterial cell suspensions using 2.5-ml syringes. Observations of the leaves were carried out by 24-48 after infiltration. The excitation wavelengths for GFP and YFP were 488 nm and 514 nm, respectively. The GFP and YFP signals were detected at 495-510 nm and 550-593 nm, respectively.

RESULTS

XGD1 At5g33290 Encodes a putative glycosyltransferase

The locus of AT5g33290 has an open reading frame made up of 4 exons (Fig. 1). The gene structure is supported by isolation of full length cDNA clones (e.g. GenBank accession BX831739 originating from Genoscope - Centre National de Sequencage : BP 191 91006 EVRY cedex - FRANCE). The encoded protein of 500 amino acids is calculated to have a polypeptide molecular mass of 56.6 kD. The protein is predicted to be targeted to the secretory pathway and to have a single transmembrane helix from residue 9 to 31, which is typical for a type II membrane protein targeted to Golgi vesicles. The protein has seven potential sites for N-linked glycosylation, but only four sites are predicted to be glycosylated. The protein is predicted to be an inverting glycosyltransferase by sequence similarity to other CAZy Family 47 proteins.

T-DNA insertion lines

One T-DNA insertion line was obtained from the Syngenta SAIL collection and another one from SALK (Fig.1). Based on sugar composition analysis we have designated the mutants *xylogalacturonan deficient 1-1* (*xgd1-1*) and *xylogalacturonan deficient 1-2 (xgd1-2)*, respectively. Homozygote lines were identified by PCR. For *xgd1-1* the PCR yielded a product of expected size but in case of *xgd1-2* the insert proved to be located approximately 400 bp downstream of the original position determined by Syngenta. Consequently, the T-DNA insertion of *xgd1-2* is located 90 bp upstream of the stop codon of *XGD1*, as verified by sequencing, leading to a substitution of the last 30 amino acids of the XGD1 protein with 43 residues originating from the T-DNA insert sequence. This substitution alters the highly conserved C-terminal region expected to be part of the catalytic region of the enzyme.

Expression analysis of *XGD1*

The two independent T-DNA insertion lines were investigated using Real-Time RT-PCR. For *xgd1-1* no transcript was detected but for *xgd1-2* the disruption of the gene did not lead to any significant destabilization of the *xgd1-2* mRNA (Table I). Based on the data available in the GENEVESTIGATOR database (Fig. 2) we analyzed the expression pattern of *XGD1* in stems, adult leaves, juvenile leaves and roots by Real-Time RT-PCR (Table I). *XGD1* is most highly expressed in adult leaves and at twice the level found in juvenile leaves. In stem and roots the level is 8,2% and 5,4%, respectively, compared to the level in adult leaves.

T-DNA insertion in *XGD1* causes a decrease in cell wall (RG-I) xylose

The effect of no *XGD1* transcript (*xgd1-1*) and altered *XGD1* transcript (*xgd1-2*) was studied by determination of the monosaccharide composition for cell wall material isolated from adult leaves obtained from *xgd1-1*, *xgd1-2* and *qrt* (Fig.3). A reduction in the xylose content was observed for both *xgd1-1* and *xgd1-2* whereas none of the other monosaccharides were altered. Neither the *xgd1-1* plants nor the *xgd1-2* plants displayed any visible growth phenotypes compared to *qrt* and wild type. To investigate the specific cell wall polymer affected by the lack of *XGD1* transcript (*xgd1-1*) we first focused on the major xylose containing cell wall polymers xylan and xyloglucan. However, antibodies against xylan indicated no difference in content of this polymer in *xgd1-1* (data not shown). The unaltered levels of fucose (Table II) as well as antibodies against xyloglucan indicated that xyloglucan was also not affected (data not shown). Therefore we isolated RG-I, performed monosaccharide composition analysis (Table II Panel A) and subsequently also subjected this polymer to linkage analysis (Table II Panel B). The monosaccharide composition of RG-I revealed a clear reduction in xylose in *xgd1-1* compared to RG-I from *qrt*. Xylose could only be detected as terminal residues in RG-I of *qrt* (Table II Panel B) and this specific linkage was reduced from 4,6 mole% in *qrt* to 1,1 mole% in *xgd1-1*. No other linkage types were reduced to this extent in *xgd1-1*.

An interesting observation is that the GalA/rhamnose ratio of RG I is reduced in *xgd1-1* (Table II Panel A) compared to *qrt*. RG I was generated by digestion of purified cell wall material with pectin methylesterase and endopolygalacturonase. Endopolygalacturonase specifically digests unsubstituted homogalacturonan, hence, any missing xylose substitution of the homogalacturonan backbone would make it more susceptible to degradation by the enzyme resulting in a reduced GalA/rhamnose ratio.

Decrease in xylose is due to a decreased content of xylogalacturonan

The highly decreased content of terminal xylose residues in RG I isolated from *xgd1-1* indicated that XGA was the affected polymer. To compare the presence of XGA in the cell wall of *qrt* and *xgd1-1* plants pectin was extracted. The pectic extract was treated with XGH and subsequently analyzed by HPAEC (Fig. 4) and MALDI-TOF MS (results not shown). As shown in Figure 4, six different oligosaccharides were identified in the XGH treated pectin fraction obtained from *qrt* plants whereas no oligosaccharides were detected from *xgd1-1*. The main product from *qrt* was $GalA_3Xyl$, while GalA, GalAXyl, $GalA_2Xyl$, $GalA_5$, and $GalA_6$ oligomers were formed in minor quantities. Also four peaks (A, B, C, and D at retention times around 44 min, 46.5 min, 49 min and 51 min respectively) were found. These peaks A, B, C and D may correspond to $GalA_7$, $GalA_8$, $GalA_9$, and $GalA_{10}$ respectively, as the corresponding masses (m/z) for these oligosaccharides were detected by MALDI-TOF MS (results not shown). Although it cannot be excluded that XGH-resistant XGA exists in the pectic extract from *xgd1-1*, the XGA content in *xgd1-1* is clearly reduced compared to *qrt* due to the absence of *XGD1* transcript.

Antibody labeling

The alterations in XGA content as well as the distribution of partially methyl-esterified homogalacturonan was investigated by indirect immunofluorescence labeling using the XGA specific antibody LM8, raised against XGA isolated from pea (*Pisum sativum* L.) testae, and JIM5 (partially methyl-esterified homogalacturonan), respectively. Surface labeling of roots indicated no differences in the level of the XGA epitope recognized by LM8 between *qrt* and *xgd1-1* (Fig.5 a and b). In siliques, the LM8 epitope was restricted to certain cells in the septum between locules , and again no differences in LM8 labeling were observed between *qrt* and *xgd1-1* (Fig. 5 c and d). As seen from Fig.5 (e,f,g and h) the tissue and cell wall structure (in terms of JIM 5 epitope and calcofluor staining, respectively) is unaltered in *xgd1-1* compared to *qrt*. The LM8 epitope (highly substituted XGA) is unaffected by the *xgd1* genotype.

Complementation of the mutant phenotype

By transformation of *xgd1-1* plants, with a construct carrying *XGD1* under control of the CaMV 35S promoter, the reduced xylose content of the cell wall material was restored to wild type level. Transformation of *qrt* plants with the same construct had no effect on the monosaccharide composition (Table III). These experiments show that the *XGD1* gene can complement the *xgd1* T-DNA mutant phenotype. Furthermore, introduction of a construct expressing a XGD1 protein extended at the C-terminal with a combined c-myc-6xHis affinity tag adding a total of 16? amino acids into the *xgd1-1* plants also complemented the *xgd1* phenotype i.e. the xylose content was again restored to wild type level (Table III). However, complementation was not obtained when the *xgd1-1* plants were expressing an N-terminal truncated version of the XGD1 protein lacking the signal anchor, the transmembrane domain and part of the stem region (amino acid 1-160).

Subcellular localization of XGD

In order to study the subcellular localization of XGD1, a translational fusion of XGD1 and YFP were generated and its localization was studied in *N. benthamiana* by confocal laser scanning microscopy. The control leave specimens showed a uniform distribution of YFP signal along the cell periphery as well as in nuclei, which confirm that YFP, when expressed alone, localizes in the cytosolic and nucleus compartment (Fig. 6a). In contrast, the leaves expressing the XGD-YFP fusion protein accumulate the YFP signals in small oval dots interspersed across the cells (Fig. 6b). In order to obtain further insights into the subcellular localization of XGD-YFP, coexpression of this and the known marker proteins were carried out. Fig. 6c and e show confocal images of a leaf co-expressing XGD-YFP and GFP-HDEL (the ER marker), respectively, and Fig. 6d shows the superimposition. It is noteworthy that XGD-YFP and GFP-HDEL showed distinct distribution patterns, the former showing an interspersed dot distribution while the latter showing a uniform distribution along the cell periphery super with spider-web-like meshwork, which is characteristics of ER (Latignhouwers *et al.*, 2005). Fig. 6d and f show confocal images of a leaf co-expressing XGD-YFP and STtmd-GFP (Golgi marker), respectively and Fig. 6h shows the nearly perfect superimposition of the two signals. Taken together, these results demonstrate that XGD-YFP fusion protein localizes in the Golgi apparatus in *N. benthamiana*.

DISCUSSION

XGD1 and paralogs of GT 47 are all predicted to be membrane bound glycosyltransferases

According to the nomenclature used at http://cellwall.genomics.purdue.edu XGD1 (At5g33290) belongs to subgroup C of CAZy GT Family 47. Nomenclature and grouping has been modified compared to the earlier described (Li *et al.*, 2004). Subgroup C includes 7 members of which only 2?? of the sequences are supported by full length cDNA clones. Previous annotation of At5g33290 predicted the protein to be soluble without a transmembrane domain (Li *et al.*, 2004). However, the longer cDNA used in this work suggest that XGD1 is a type II membrane bound protein. Four of the other members of subgroup C (At3g42180, At5g20260, At5g11130 and At5g25310) had also been predicted to be soluble proteins according to the original annotation (Li *et al.*, 2004). However, thorough analysis of the gene structure as well as the translated sequence of the other members of this group we have been able to reannotate these genes and suggest that they all encode type II membrane proteins. Furthermore, all genes within this group share the same intron-exon structure according to our annotation. The relatively large intron 2 varying in size; a potential alternative start codon in exon 3, and the lack of supporting cDNA sequence information could be the explanation for the previous annotation difficulties within this group. However, the alignment of the protein sequences based on the predicted sequences in this work leaves little doubt that all genes encode type II membrane bound proteins. Furthermore, alignment of these Arabidopsis sequences and their homologs from rice strongly supports our conclusion.

T-DNA insertion in XGD1 causes a decrease in xylogalacturonan

Interestingly, both of the analyzed T-DNA insertion lines (*xgd1-1* and *xgd1-2*) show an identical decrease in cell wall xylose, even though only *xgd1-1* is a true mRNA knock-out line i.e. no transcript present.

The T-DNA insertion in *xgd1-2* presumably results in a chimeric protein altered in the C-terminal and catalytic part of the protein, which, if stable, is an inactive enzyme. The expression pattern of *XGD1* was analyzed by Real-Time RT-PCR in stems, adult leaves, juvenile leaves and roots. The relative expression pattern between adult leaves, juvenile leaves and stem tissue is in good agreement with the data available in GENEVESTIGATOR (Zimmermann *et al.*, 2004) although the level in stem tissue is somewhat higher compared to our observation. The expression level in roots, however, was found to be much lower compared the level reported in GENEVESTIGATOR, where it is reported to be almost equal to the level in juvenile leaves. This discrepancy could be due to the growth conditions used in our experiment. Roots for RNA preparation were grown hydroponicly, which is an artificial system. Includingly, there can also be a possible interference from At5g42180, as this gene is not present on the Affymetrix-chip; in fact only one of the other members of subgroup C are present as well. The GENEVESTIGATOR database also reports on the elevated expression of XGD1 when plants have been subjected to pathogens like *Botrytis cinerea*, *Phytophtora infestans* and *Pseudomonas syringae* suggesting a function of XGA in resistance to pathogen attack.

The decrease in cell wall xylose was observed for both *xgd1* alleles. We continued our investigations focusing on *xgd1-1* in our attempts to discover the affected polymer. The levels of the major xylose containing polymers xylan and xyloglucan were by dot blot analysis found to be unaltered as compared to *qrt*. However, when the xylose content of the purified RG I was analyzed specifically, we observed a significant difference: xylose was present in *qrt* while clearly reduced in *xgd1-1*. Interestingly, not only the xylose content but also the GalA content was reduced in RG I prepared from *xgd1-1* cell wall material, indicating extended degradation by the polygalacturonase compared to wild type. Furthermore, we found the xylose residues to be present as terminal residues only. Both observations point to a reduction in xylogalacturonan content in *xgd1-1* as a consequence of no *XGD1* transcript.

Xylogalacturonan has previously been shown to be present in Arabidopsis based on digestion of pectic extracts with xylogalacturonan hydrolase (Zandleven *et al.*, In preparation). Cell wall material isolated from adult leaves from *xgd1-1* and wild type (*qrt*) was analyzed for the presence of xylogalacturonan using the very same procedure. While XGA oligomers could be detected in the wild type extract, no XGA oligomers were detected in the *xgd1-1* extract. A subsequent analysis of the distribution of xylogalacturonan by use of the xylogalacturonan specific monoclonal antibody LM8, which has previously been shown to label part of the root tip in Arabidopsis (Willats *et al.*, 2004) resulted in an interesting observation. A detailed immunofluorescence analysis of the Arabidopsis plant showed labeling in root tip and very distinct labeling in certain cells in the septum between locules in the siliques in both *qrt* and *xgd1-1*. The LM8 antibody did not give rise to any labeling in adult leaves in the wild type where the *XGD1* gene is most highly expressed (data not shown). The distribution in adult leaves of partially methyl-esterified homogalacturonan (JIM 5 epitope) as well as the overall cell shape and tissue structure as visualized by calcofluor staining did not reveal any differences between *qrt* and *xgd1-1*. It has previously been suggested that LM8, which has been raised against XGA isolated from pea (*Pisum sativum* L.) testae, binds to a highly substituted epitope of XGA possibly substituted with a disaccharide of xylose that is not susceptible to cleavage by an exopolygalacturonase or the xylogalacturonan hydrolase used in this work. Therefore, even though there is no detectable XGA present in *xgd1-1*, when using the xylogalacturon hydrolase approach, the LM8 immunofluorescence labeling data indicate the presence of highly substituted XGA in tissues presumably unaffected by the *xgd1* genotype. Therefore, our results suggests the presence of two different types of XGA in Arabidopsis, a low substituted form, synthesized by XGD1, carrying single xylose substitutions which is generally present in all green tissues (Zandleven *et al.*, In preparation) and a highly substituted form which is only present in very distinct parts of the plant - the latter form being highly resistant to enzymatic hydrolysis.

Function and localization of XGD1

Given the effect of XGD1 on the XGA content of Arabidopsis cell walls, we hypothezise that XGD1 is involved in XGA biosynthesis most likely as a xylosyltransferase transferring xylose onto galacturonic acid. Pectin biosynthesis, or at least part of it, is believed to occur in the Golgi apparatus (Levy S and Staehelin 1992). Co-localization of XGD1, studied as a XGD1-YFP fusion protein, with the known Golgi marker STtmd-GFP (Boevink *et al.*, 1998) (Fig. 6) further supports our hypothesis that XGD1 is involved in pectin biosynthesis. The observation that the N-terminal truncated version of XGD1 was unable to complement the mutant phenotype is presumed to be a consequence of the missing signal anchor and transmembrane domain which might lead to mislocalization of the protein. Even though this N-terminally truncated protein possibly could be active it would not be localized in the right subcellular biosynthetic compartment. This result suggests that the appropriate localization of XGD1 to the Golgi apparatus is crucial to the function of the enzyme. As mentioned previously, the *xgd1-2* plants, which are expected to express a chimeric protein where 30 amino acids of the C-terminal are replaced by 43 amino acids of the T-DNA, display the same xylose deficient phenotype as *xgd1-1*. These 30 amino acids, which have been substituted in *xgd1-2*, are part of a highly conserved region among all genes within GT 47 subgroup C in both Arabidopsis and rice. Therefore it is plausible that this region is important with respect to catalytic activity. Interstingly, the C-terminal tagged protein (myc6His) was able to complement the mutant phenotype indicating that extension of the C-terminal does not interfere significantly with catalytic activity. Assessment of the biochemical activity of XGD1 *in vitro* still remains. We expect XGD1 to be a xylosyltransferase catalyzing the transfer of xylose from UDP-xylose to a galacturonic acid oligomer. Whether the xylosyltransferase activity requires the simultaneous elongation of the galacturonic acid backbone, like the arabinoxylan arabinosyltransferase (Porchia *et al.*, 2002), is also an open question.

The fact that two types of XGA are present in Arabidopsis suggests that one or more of the XGD1 paralogs are involved in XGA biosynthesis as well. We are currently in the process of analyzing T-DNA mutants of some of these genes.

Table I Relative expression analysis of *XGD*, expression in adult leaves is set to 100. Genevestigator data is recalculated from figure 2.

Tissue	Genevestigator	*XGD1*	*xgd1-1*	*xgd1-2*
Stems	15	8,2	n.d.	
Adult leaves	100	100,0	<0,1	103
Juvenile leafs	56,8	48,3	<0,1	
Roots	54,5	5,4	n.d.	

n.d. : not detectable

Table II. Linkage Analysis of RG I Isolated from adult leaves from wild type (*qrt*) and *xgd1-1*. Data are expressed as mol % except the GalUA / Rha ratio which is the molar ratio. A reduction in t-Xyl in *xgd1-1* could be observed in both the monosaccharide composition and in the linkage analysis.

	qrt	*xgd1-1*
Monosaccharide composition		
Fuc	2,8	3,4
Rha	18,0	21,2
Ara	28,1	27,4
Gal	12,0	15,3
Xyl	4,6	1,2
GalUA	34,5	31,4
Total	100	100
GalUA / Rha	1,92	1,48
Linkage analysis		
2-Rha	15,6	12,9
2,4-Rha	8,4	10,5
t-Xyl	4,6	1,1
t-Gal	7,4	9,0
4-Gal	8,9	12,8
3-Gal	3,4	3,1
6-Gal	3,4	2,4
3,6-Gal	5,4	4,3
3,4-Gal	1,5	1,2
4,6-Gal	0,7	1,0
t-Ara*f*	4,9	5,9
5-Ara*f*	17,7	18,6
2,5-Ara*f*	3,0	3,4
2,3,5-Ara*f*	2,7	4,1
t-Fuc	0,7	0,8
Total	88,3	91,1

Table III Complementation of the *xgd1* sugar composition phenotype

Plant genotype	Relative Xyl content to *qrt*[1]
qrt + pPZP221[1]	1.00±0.05 A
qrt + p35S::XGD1	1.04±0.14 A
xgd1 + pPZP221	0.84±0.06 B
xgd1+ p35S::XGD1	1.02±0.08 A
xgd1+ p35S::XGD1-Myc-6-His	1.00±0.07 A
xgd1+ p35S::ΔN-XGD1	0.84±0.06 B

[1]Mean±st.dev, n=5, numbers followed by the same letter are not significantly different (p>0,05).
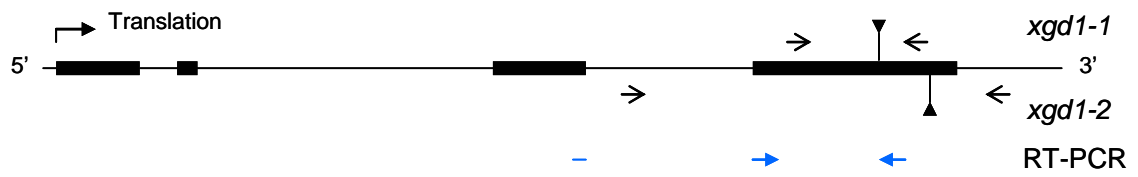
Figure 1 The gene structure of *XGD1* with introns (slim lines) and exons (thick lines). Primers for verification of T-DNA inserts are shown as black arrows. Primers for RT-PCR are shown as blue arrows.
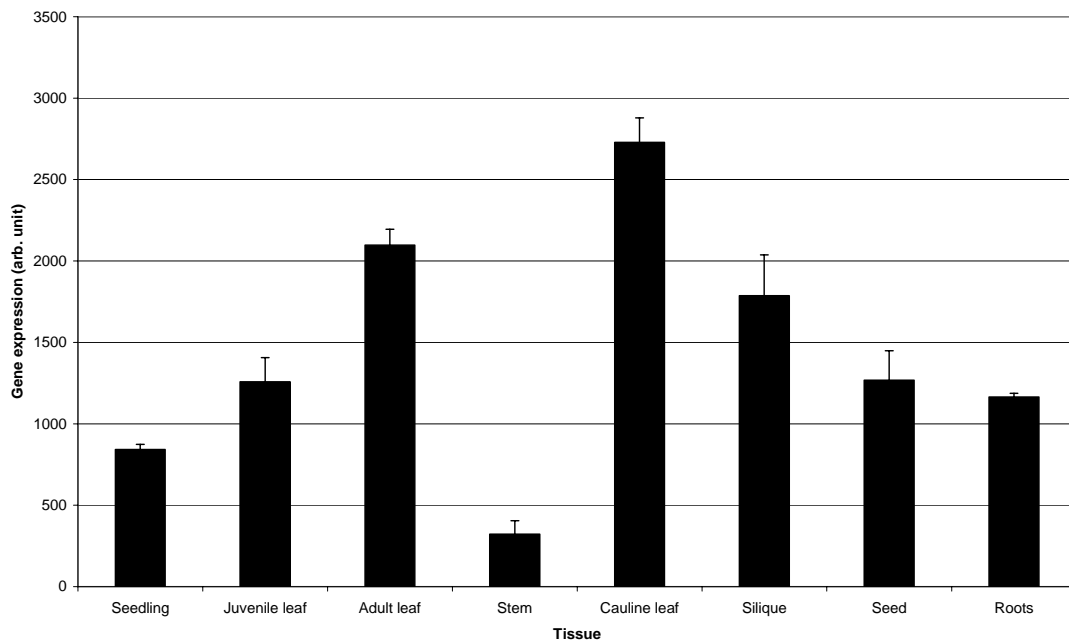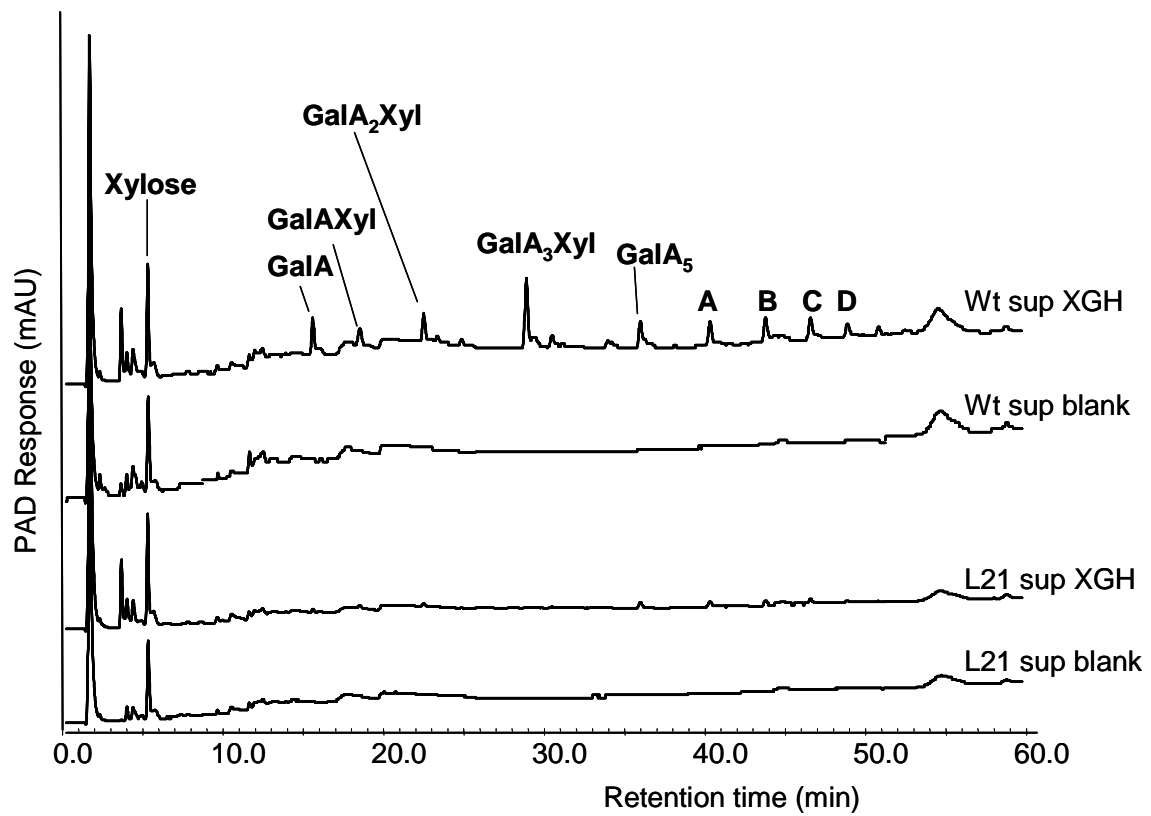

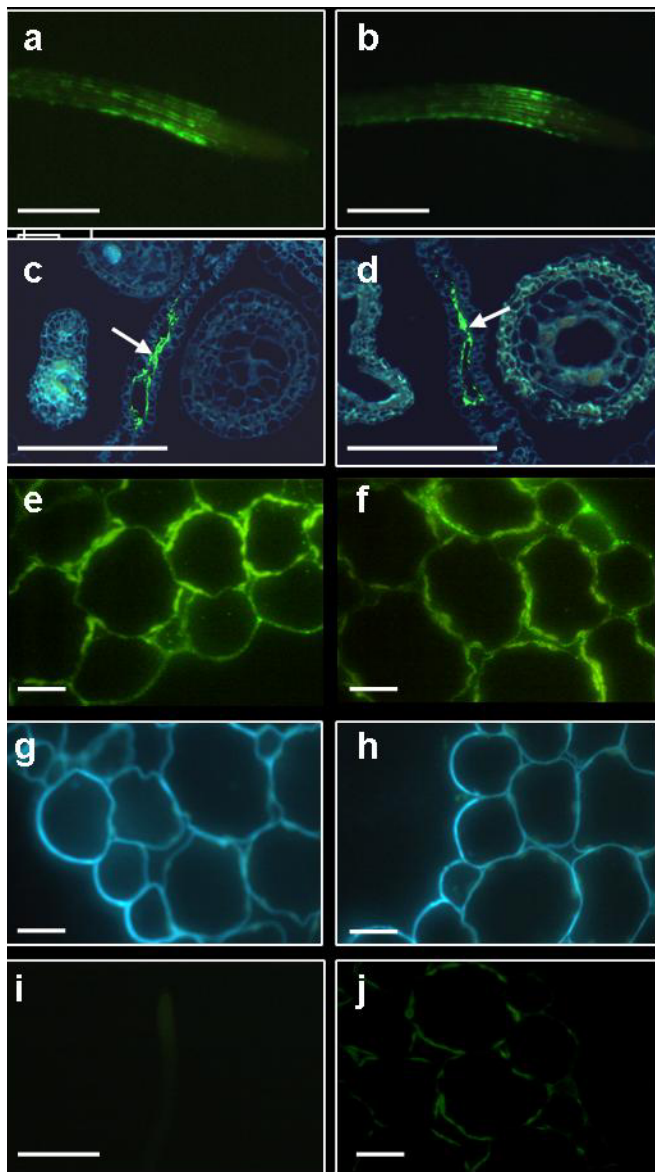**Figure 2** The relative gene expression of *XGD1*. Data from Genevestigator (Zimmerman *et al.*, 2004).
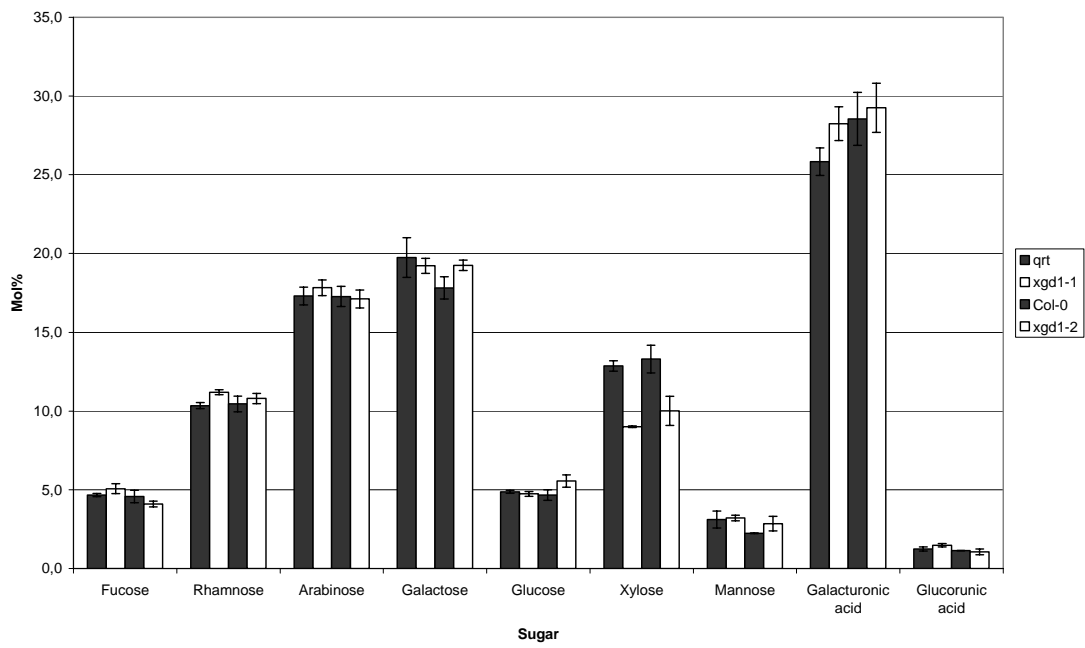
**Figure 3** The sugar composition of alcohol insoluble residues from adult leaves.

Figure 4 The HPAEC-PAD response from oligomers solubilised by treatment with XGH. Blank controls are included.

Figure 5 Indirect immunofluorescence labelling of *qrt* (b,d,f and h) and *xgd1-1* (a,c,e and g). Labelling was performed on intact roots (a and b) and transverse sections of resin embedded siliques (c and d) and leaves (e –h). Roots and siliques were labelled with an antibody with specifictity for xylogalacturonan (LM8) and leaf sections with an antibody with specificity for partially methyl-esterified homogalacturonan (JIM5, e and f) and calcoflour (g and h). Surface labelling of roots indicated no differences in the level of the xylogalacturonan epitope recognised by LM8 between *qrt* and *xgd1-1*line 21. In sliques, the LM8 epitope was restricted to certain cells in the septum between locules (indicted by arrows), and again no differences in LM8 labelling were observed between qrt and line 21.

Similary, labelling of leaf sections with JIM5 (e and f), and staining with calcoflour (g and h) were essentially the same qrt and line 21. Antibody labelling was essentially absent in no prmary antibody controls of surface labelled roots (i) and leaf sections (j). Scale bar =100 µm (a, b, c, d, i), 10 µm (e, f, g, h, j).
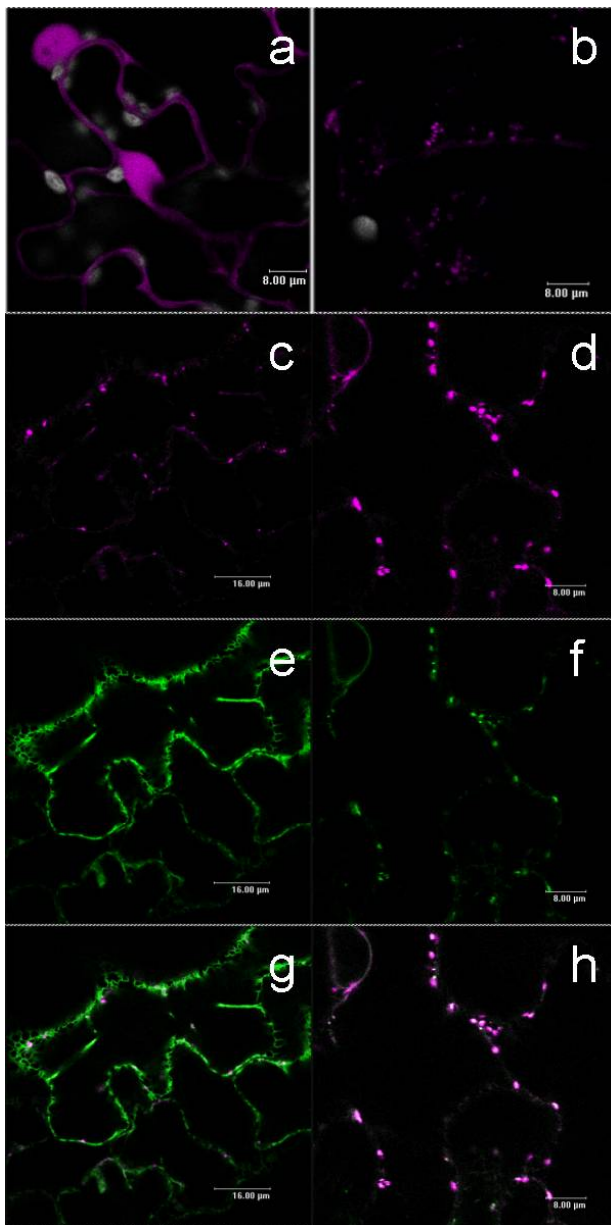
Figure 6 Subcellular localization of XGD-YFP in N. benthamian analyzed by confocal scanning laser microscopy. Photographs were taken for leaf specimens expressing YFP (control), a, and XGD-YFP, b. Coexpression of XGD-YFP and an ER marker (GFP-HDEL) are shown in c, e, and g where c and e represent YFP and GFP signals, respectively, while g represent the merge of the two signals. Likewise, coexpression of XGD-YFP and a Golgi marker (STtmd-GFP) are shown in d, f, and h where d and f represent YFP and GFP signals, respectively, while h represent the merge of the two. Magenta, green, and grey represent YFP, GFP, and chloroplast autofluoresence signals, respectively.

Overlap of the YFP and GFP signals appear white (h). Excitation wavelengths for GFP and YFP were 488nm and 514nm, respectively. GFP and YFP emission were detected between 495-510nm and 560-59xnm, respectively. OD600nm of Agrobacterium inoculum was 0.05.

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Appendix

This appendix contain co-author statements