University of Potsdam

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Pectin: New Insights from an Old Polymer through Pectinase-Based Genetic Screens

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Abbreviations

AA	Alditol Acetate
ABRC	Arabidopsis Biological Resource Center
AD	Arbitrary degenerate
AGA	Apiogalacturonan
AIR	Alcohol Insoluble Residue
ANOVA	Analysis of variance
Ara	Arabinose
ATTED-II	Arabidopsis thaliana trans-factor and cis-element prediction database-II
BAR	Bio-Array Resource
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAZY	Carbohydrate active enzyme
cDNA	complementary DNA
CLG	Cross-linking glycan
Col	Columbia
CTAB	Cetyl trimethylammonium bromide
DHB	Dihydroxybenzoic acid
DM	Degree of methyl-esterification
DP	Degree of polymerisation
DUF	Domain of unknown function
EMS	Ethyl methanesulfonate
endoPG	Endopolygalacturonase
FITC	fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC/MS	Gas chromatography/Mass spectrometry
GFP	Green fluorescent protein
Glc	Glucose
GT	Glycosyltransferase
GUS	ß-glucuronidase
HBG	Highly branched galacturonan
HG	Homogalacturonan
HR	Hypersensitive reaction
JIM	John Innes Monoclonal
kb	kilobase pair
KDO	2-keto-3-deoxyoctulosonic acid
LB	Left border (T-DNA)
LD	Linkage disequilibrium
Ler	Landsberg erecta

LM	Leeds Monoclonal
LOD	Logarithm of odds
LOPIT	Localization of Organelle Proteins by Isotope Tagging
LRR	Leucine rich repeat
mAb	monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MFS	Major Facilitator Superfamily
MPSS	Massively parallel signature sequencing
NCBI	National Center for Biotechnology Information
NIL	Near-isogenic line
NMR	Nuclear Magnetic Resonance
OGA	Oligogalacturonide
OLIMP	Oligosaccharide mass profiling
PACE	Polysaccharide analysis by capillary gel electrophoresis
PAE	Pectin acetylesterases
PCR	Polymerase chain reaction
PL	Pectate lyase
PME	Pectin methyl esterase
PMT	Pectin methyl transferase
PTB	Polypyrimidine tract-binding protein
QTL	Quantitative trait locus
RG	Rhamnogalacturonan
Rha	Rhamnose
RIL	Recombinant Inbred Line
ROS	Reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
SAH	S-adenosyl-L-methionine
SAM	S-adenosyl-L-methionine
SBS	Sequencing by synthesis
SEC	Size exclusion chromatography
SNP	Single-nucleotide polymorphism
SSLP	Simple sequence length polymorphism
ST	Syalil Transferase
TAIL-PCR	Thermal asymmetric interlaced polymerase chain reaction
TAIR	The Arabidopsis Information Resource
T-DNA	Transfer-DNA
TM	Trans membrane domain
TMS	trimethylsilyl
UA	Uronic acid
UDP	Uridine diphosphate
WT	Wild type
WT	wild-type
XGA	Xylogalacturonan
Xyl	Xylose
YFP	Yellow fluorescent protein

1 Introduction

1.1 The Plant Cell Wall

The plant cell wall encircles each cell in a plant. The presence of rigid cells walls contributes to the sessile life-style of higher plants and impacts differences in nutrition, digestion, growth, reproduction, osmoregulation, defense mechanisms, intercellular communication, and morphology (Alberts, B. 1989). The cell wall should possess durability to counteract mechanical loadings (up to 100 MPa), and at the same time be able to extend during cell development to allow for cell expansion (Cosgrove, D. J. 1993). When a cell grows, the polysaccharide network is remodeled; this process involves breaking and formation of covalent and non-covalent bonds (Fry, S. C. 1986). Cells can elongate many times their size by loosening but not weakening the cell wall. The cell wall participates in defining the direction and rate of cell elongation and regulating cell volume, in maintaining and determining cell shape which ultimately makes it responsible for the plant architectural design and controlling plant morphogenesis (Somerville, C. et al. 2004). This organelle is also involved in controlling mechanical strength and water pressure, in forming water- and an ion-conductivity, in cell recognition, stress response and signaling (Cosgrove, D. J. 2005, Darvill, J. E. et al. 1980, Nothnagel, E. A. et al. 1983). The cell wall has a role in several stage-specific processes: maintaining seed germination, fruit ripening, and abscission of leaves (Brummell, D. A. et al. 2004, Gonzalez-Carranza, Z. H. et al. 2002).

Cell walls possess a high structural complexity of a supramolecular structure which includes numerous polymers with diverse and complex structures (see fig. 1.2). Recently, the molecular structure of the cell wall components has been decoded, primarily for the polysaccharides, which are among the most complex known molecules. A considerable quantity of polysaccharide-acting enzymes is present in the cell wall, including various hydrolases, lyases, transglycosylases, esterases and expansins; there are also structural proteins (extensins, arabinogalactan proteins, proline- and glycine-rich proteins) that contribute to wall assembly, architecture and growth (Jamet, E. et al. 2006, Jamet, E. et al. 2008).

There are three distinct regions of the cell wall: the middle lamella, the primary cell wall and the secondary cell wall. All plant cells have middle lamella and a primary wall while the

secondary wall is present only in certain cell types. The middle lamella is the outmost layer of the wall that forms the interface between the primary walls of adjacent cells, binding them together. The primary wall is formed in the cell plate during cell division and is rapidly deposited during cell expansion and active growth (see thin cell walls weakly labeled for cellulose in fig.1.1). The secondary cell wall is formed in some cells when they stop growing and is deposited between the primary wall and the plasma membrane (see thick cell walls strongly labeled for cellulose in fig.1.1).

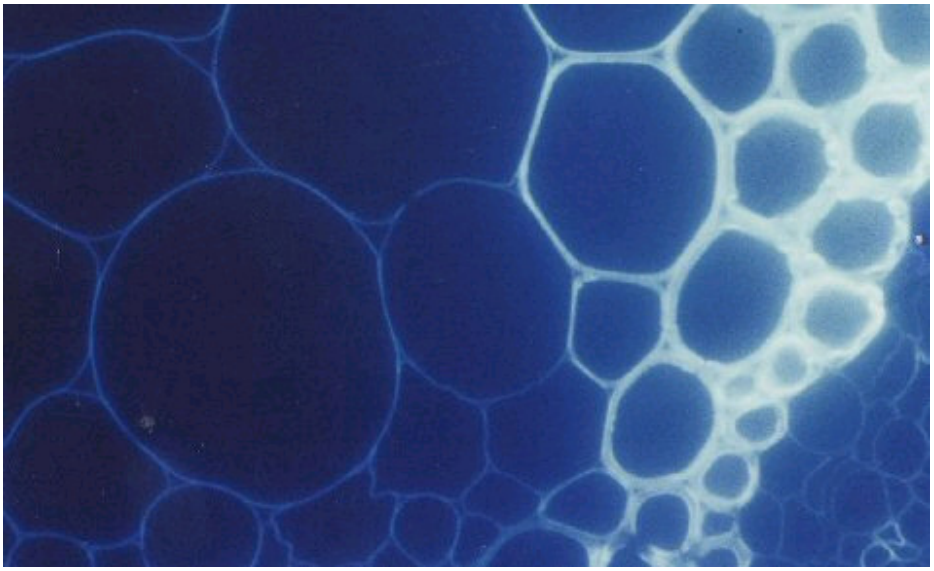

Figure 1.1. Section of Arabidopsis floral stem showing Calcofluor White staining of cellulose. The secondary walls of the sclerified parenchyma are thick and have high cellulose content, (strong Calcofluor White staining) while the primary walls of the surrounding cells are thin and have less cellulose (weaker staining). Image by Paul Knox (www.plantcellwalls.net).

1.1.1 Structure and Composition of the Plant Cell Wall

One basic component of all primary cell walls is water. The cell wall matrix is largely hydrated and consists of up to 70% (v/v) of water (Monro, J. A. et al. 1976). Water contributes for some of the wall properties, providing flexibility and extensibility of this matrix that mimics a dense water gel (Cosgrove, D. J. 1997). One important characteristic of the cell wall is its pH value, which is between 4 and 5 (Schopfer, P. 1993). Other components of the wall include polysaccharides, phenolic compounds (lignin, present in secondary cell walls), lipids (cutin and suberin, present in special cell types) and proteins (Heredia, A. et al. 1995).

Three types of polysaccharides are present in the cell wall: cellulose, cross-linking glycans (CLGs, formerly named hemicelluloses) and pectins (see Table 1.1.). Cellulose is the primary cell wall polysaccharide and the most abundant organic polymer on Earth. This unique polysaccharide comprises linear homoglucan chains composed purely of beta-1,4 linked D-glucose residues. Each chain has a variable degree of polymerization (DP) of up to 6.000 and 14.000 in primary and secondary walls, respectively (Fincher, G. B and Stone, B. A. 2004). Three dozen or more chains are interconnected by hydrogen bonds in highly ordered parallel arrays to make crystalline microfibrils of about 3 nm in diameter in primary walls and 5–10 nm in diameter in secondary walls (green ribbons on fig. 1.2) (Fincher, G. B and Stone, B. A. 2004, Jarvis, M. 2003).

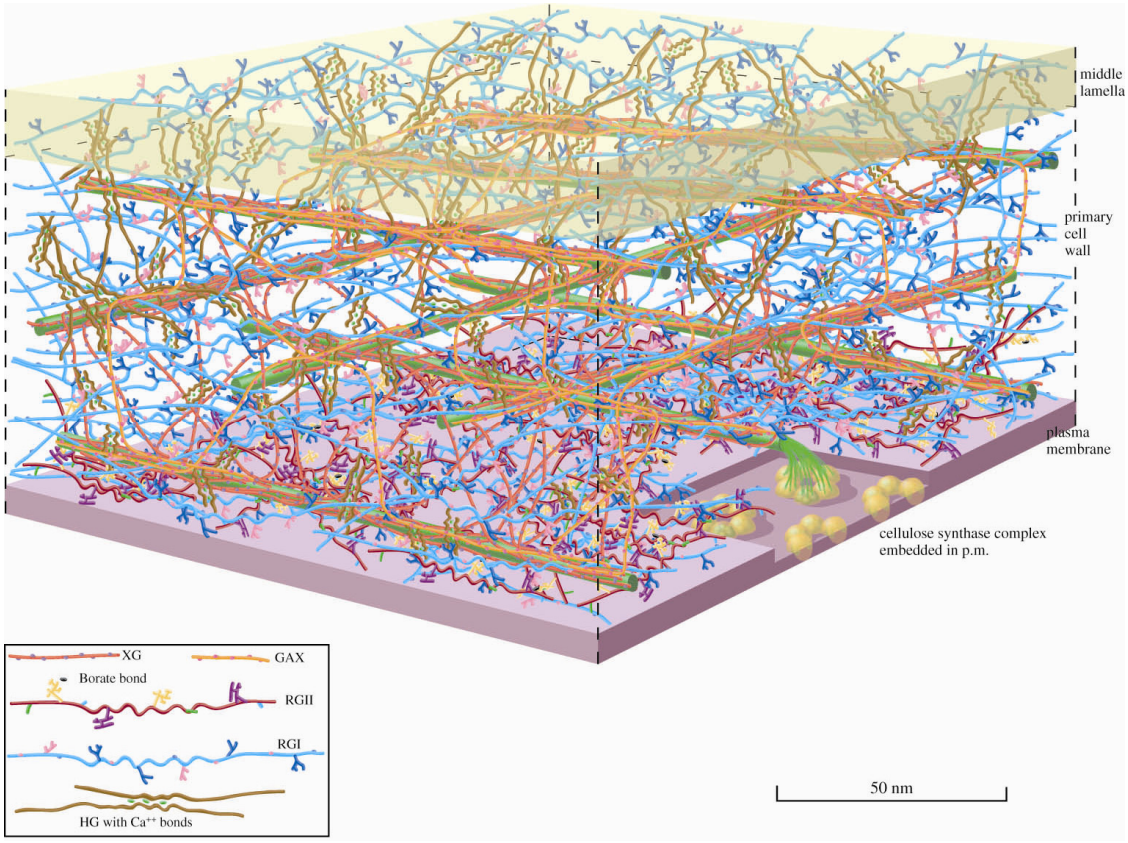

Figure 1.2. Scale model of a portion of the plant cell wall. The orthogonally arranged layers of cellulose microfibrils (green) and CLG [here xyloglucan (XG) and glucuronoarabinoxylan (GAX)] form a network, which is embedded in a pectic matrix containing homogalacturonan (HG), rhamnoglacturonan I (RG-I) and rhamnogalacturonan II (RG-II). The middle lamella is rich in pectin and cements adjacent cells together. (Somerville, C. et al. 2004)

Cross-linking glycans (CLGs) are defined by their connection with cellulosic microfibrils: they are a heterogeneous group of branched polysaccharides that bind tightly to the surface of each cellulose microfibril and thereby help cross-linking microfibrils into a complex network. The classification of these polymers is based on the type of sugar that is predominant in their backbone (glucose, xylose, or mannose); therefore they are classified as either glucans, xylans or mannans. The backbone sugar molecules are responsible for forming hydrogen bonds with the surface of cellulose microfibrils, cross-linking them in the process. Both the backbone and the side-chain sugars vary according to the plant species and its stage of development (Alberts, B. 1989).

1.2. Pectins

The cellulose microfibrils and the CLGs form a network which is embedded in a matrix of pectin polymers. The term "pectin" originates from the Greek word “pektikos” meaning to congeal or solidify, which describes the basic properties of these supramolecules to form gels with calcium ions (Pilnik, W 1990). Pectin was first described by Henry Braconnot in 1825, when he also predicted that it would have important functions in all plants (Braconnot, H. 1825).

The term ‘pectin’ is somewhat misleading, since it implies one entity. In fact it comprises a family of oligo- and polysaccharides that have common features, but are structurally diverse (Ridley, B. L. et al. 2001). This suite of polymers are one of the most structurally complex and chemically diverse molecules of biological origin, involved in a variety of functions in the plant (Levy, S. and Staehelin, L. A. 1992, Ridley, B. L. et al. 2001). All pectic polysaccharides have one feature in common: they are characterized by a high content of D-galacturonic acid (GalA). Because of the GalA, the cell wall has a negative charge and behaves as an insoluble polyanion (Nari, J. et al. 1991). The carboxylic groups of the GalA units can bind bivalent metal ions (e.g. Ca2+ and heavy metals) thus creating local pH dynamics (Manunza, B. et al. 1998). Furthermore, pectins define cell wall porosity, ion transport, and water retention, and they are involved in defense mechanisms against infections, wounding and stress (Ridley, B. L. et al. 2001). One can consider pectin to be the hydrophilic cement between cellulose microfibrils, preventing their collapse at falling turgor pressures (Chanliaud, E. et al. 2002). In current models of primary cell walls, pectic polysaccharides are thought to form a

single covalently linked network, although a standard classification as individual components is still maintained (Mohnen, D. 2008, Vincken, J. P. et al. 2003).

1.2.1 Chemical structure of pectin

There are 3 major structural classes of pectic polysaccharides that are thought to be found in all primary cell walls. These polysaccharides are homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II), all containing GalA to a greater or lesser extent. Other related polysaccharides, such as xylogalacturonan (XGA) are considered as modifications and appear to have a more limited distribution (Willats, W. G. et al. 2004).

HG is the basic component of pectinaceous substances, it is a homopolymer consisting of 1,4-linked α-D-GalA and is thought to account for about 50-70% of primary cell wall pectin (Ridley, B. L. et al. 2001). HG is currently supposed to have an average degree of polymerization (DP) of 100 monomers (Prade, R. A. et al. 1999). The HG backbone can be modified by methyl esters attached to the C-6 and acetyl esters attached to the C-2 or C-3 atoms of GalA, with a complex distribution pattern (see fig. 1.3 A) (Ridley, B. L. et al. 2001). Both the degree of methyl-esterification and degree of acetyl-esterification affect the functional properties and the physical behavior of HG (Willats, W. G. et al. 2001b) . While highly esterified HG can be cross-linked by hydrogen bridges and hydrophobic forces between methoxyl groups, low esterified HG forms junction zones by Ca^{2+} cross-linking between free carboxyl groups (so called “egg box” structure, see fig. 1.3 B). Therefore the pattern of esterification determines the ratio between the two types of cross-links and defines the rheological properties of the formed network (Willats, W. et al. 2006). Recently it has been proposed that the pattern of esterification can have regular, random or blockwise distribution (See fig. 1.3. C) (Daas, P. J. et al. 2001a).

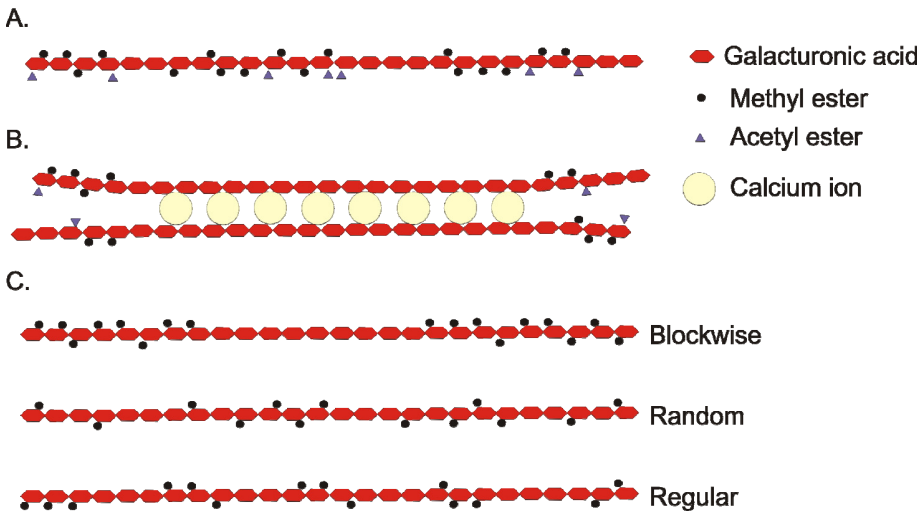

Fig.1.3. Schematic structure of Homogalacturonan (HG). A: Fragment of methyl-and acetyl-esterified HG, B: Ca^{2+}-bound "egg-box" structure of two HG chains. C: Scheme of three HG fragments with different methyl-esterifiaction pattern (blockwise, random and regular) (Daas, P. J. et al. 2001b).

Considerably less widespread are xylogalacturonan (XGA) and apiogalacturonan (AGA). XGA is found predominantly in storage tissues and reproductive organs of higher plants, and AGA in present in some aquatic monocots, such as duckweed {Hart, 1970 4302 /id;Bouveng, 1965 4350 /id;Schols, 1995 4349 /id}. These polymers are related to HG as they consist of a galacturonic acid backbone, which is substituted with side chains consisting of the single or two residues of β-D-xylopyranose or β-D-apiofuranose at the O-3 position of the galacturonic acid. The frequency of branching varies from 25 to 75 % (Schols, H. A. et al. 1995). Little is known about the function of these polysaccharides, although it has been shown that they are resistant to fragmentation by microbial and fungal EPGs.

Rhamnogalacturonan I (RG-I)

RG-I is a highly variable heteropolymer in which the backbone consists of ~ 30 residues of alternating α-(1→2)-rhamnose and α-(1→4)-galacturonic acid (Talmadge, K. W. et al. 1973). Between 20 and 80 % of the rhamnose residues can be substituted with sidechains of various lengths from 1 galactose to 50 or more residues of arabinose (arabinan), galactose (galactan), or both (arabinogalactan). Branching of the backbone occurs only at the rhamnose residue (at C-4), but the galacturonic acid residues can be methyl esterified at C-6 and acetyl

esterified at C-2 or C-3. Furthermore, neutral side chains can have esters of ferulic acid (see fig. 1.4). Oxidative coupling of these ferulate residues by the action of peroxidases produces diferulate cross-links, linking different polysaccharide chains (Fry, S. C. 1986). The types of side chains substantially define porosity of the cell wall, its charge, pH and balance of ions (Carpita, N. C. and Gibeaut, D. M. 1993). The degree of polymerization of RG-I can strongly vary, with an average of 1000 DP.

The side chains α-L-(1→5)-arabinan and β-D-(1→4)-galactan can be linear or branched. However, if side chains are present, they are (as a rule) a single residue of the same sugar as the backbone. The question of whether arabinan and galactan are always side branches of RG-I or if they can be "independent" polymers is still under debate.

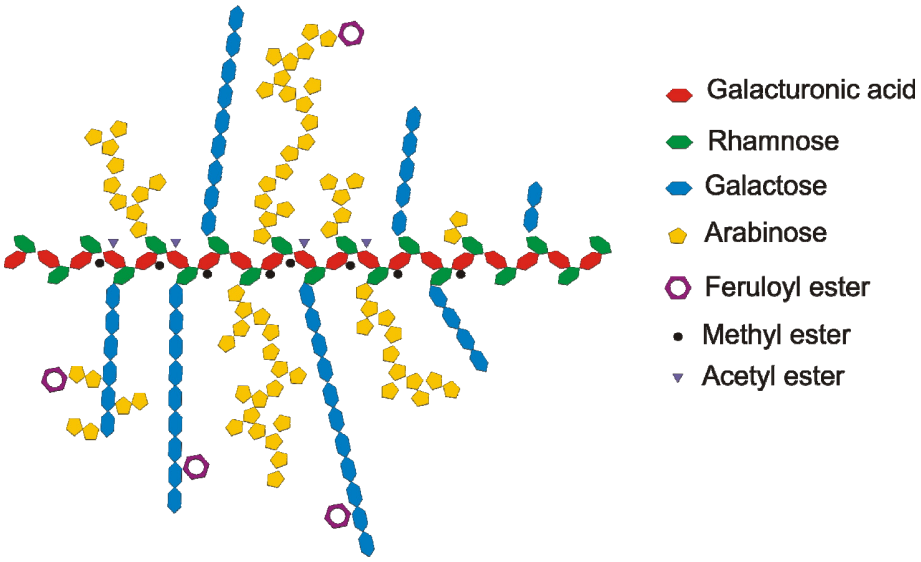

Figure 1.4. Schematic structure of RG-I represented with side chains of galactan and arabinan.

Rhamnogalacturonan II (RG-II), or highly branched galacturonan (HBG)

RG-II is the most complex polysaccharide known in living organisms so far, though the DP is quite low, approximately 60 monomers (O'Neill, M. A. et al. 1996). RG-II contains four side-chains linked to an α-(1→4)-GalA backbone, consisting of 12 different monosaccharides, some of with are rare sugars. Side chains are composed of rhamnose, arabinose in a rare pyranose form, galactose, fucose, methylfucose, methylxylose, apiose, glucuronic acid (GlcA), 2-keto-3-deoxyoctulosonic acid (KDO) – a rare monosaccharide, and aceric acid, which is only

present in RG-II (see fig. 1.5). The structure of these side chains was carefully characterized; however, the exact order of their arrangement on the backbone remains unknown. RG-II forms a homodimer linked by a boron ion. The mechanical properties of the cell wall, for example its porosity and elasticity can depend on these complexes (O'Neil et al. 1996). RG-II is considered to have highly conserved structure and it does not differ significantly amongst different plant species and tissues. Recently a novel nomenclature was proposed for RG-II, namely highly branched galacturonan (HBG), to distinguish it from RG-I. (Schols, H. A. and Voragen, A. G. J 2002).

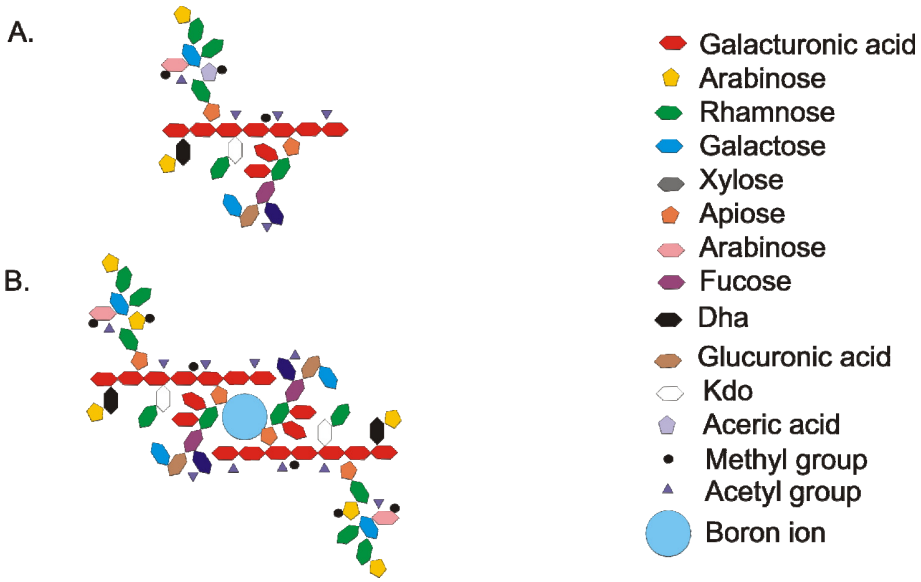

Figure 1.5. Schematic structure of RG-II. A. Monomer of RG-II B. Homodimer of RG-II with Boron.

The pectic network

The fine structure of pectin can be extremely heterogeneous between plants, between tissues, and even within a single cell wall. The degree of polymerization of various domains can vary considerably too. One polysaccharide molecule can differ from another on several levels; this includes the degree of polymerization, the presence, structure, length and branching of side chains, the presence of modifying groups (esters) and their arrangement. These varying parameters create a basis for the mosaic structures of the cell wall. It is widely accepted that it would be virtually impossible to find two identical molecules of the same polysaccharide within

the cell wall of an individual cell, at least for complex polysaccharides such as RG-I (Willats, W. G. et al. 2001a) .

A key question that still remains is how the HG, RG-I and RG-II are linked together and integrated within a network of the cell wall. The conventional model proposes that all three types are covalently linked along a linear backbone composed of HG and RG, resulting in distinct RG-I and RG-II domains. However, a different model has recently been proposed suggesting that RG-I backbone forms a core domain to which HG and neutral side chains are attached (Vincken, J. P. et al. 2003).

Interaction of pectin with other polymer systems

In current models of primary cell walls, pectic polysaccharides are thought to form a single covalently linked matrix that surrounds and embeds the cellulose-CLG network (Cosgrove, D. J. 2005). However, the notion that the pectin matrix merely coexists and only embeds the cellulose-CLG network may be an over-simplification. There is recent evidence of a direct link of specific pectic polymers to both cellulose and CLG (Cumming, C. M. et al. 2005, Zykwinska, A. et al. 2005). A recent study demonstrated that RG-I side chains have the capacity to adhere to the surface of cellulose in a manner similar to CLGs, indicating that these domains may act in cell wall assembly or extensibility (Zykwinska, A. et al. 2005).

New findings show that the cross-link of RG-I side chains with xyloglucan consists of a covalent bond that is formed intra-protoplasmically and not in the cell wall. This link is stable and contributes to cell wall assembly (Popper, Z. A. and Fry, S. C. 2008). However, the mechanism by which these bonds are formed is still elusive. There are no hetero-transglycosylases identified so far, and the data indicates that a portion of xyloglucan is directly synthesized on RG-I side chains (Abdel-Massih, R. M. et al. 2007, Popper, Z. A. and Fry, S. C. 2008).

1.2.2. Methodology used in pectin research

Biochemical and analytical chemistry methods

Various extraction and fractionation methods have been developed for studying pectin structure. These procedures usually involve the sequential extraction of alcohol insoluble

residue (AIR) to release different pectic polymers (Voragen, A. G. J et al. 1995). For example, water soluble pectins would contain polymers that are not attached to cellulose and are loose while chelator soluble fraction would yield Ca^{2+}-bound pectins, a carbonate soluble fraction would be enriched in esterified pectins, since carbonate would de-esterify the polymer (Schols, H. A. and Voragen, A. G. J 2002). Usually these extracts are fractionated by size exclusion and/or anion-exchange chromatography to produce a homogeneous purified polymer (Schols, H. A. and Voragen, A. G. J 2002). In general such purified fractions can be submitted for analysis of composition, linkage and configuration of the sugar residues, or can be digested with specific enzymes and the resulting fragments can be analyzed by capillary or gel electrophoresis, Thin layer chromatography, NMR or MS (Barton, C. J. et al. 2006, Colquhoun, I. J. et al. 1990, Doco, T. et al. 2001, Herve du, Penhoat C. et al. 1999, Schols, H. A. et al. 1995, Strom, A. et al. 2005).

Immunocytochemistry

Physicochemical analyses of pectin are fundamental to the refinement of our knowledge of pectin biology. However, these techniques do not provide the necessary information on spatial and developmental aspects of pectin structure. Currently, anti-pectin monoclonal antibodies (mAbs) are one of the best tool for studying deposition and developmental occurrence of pectin in relation to the cell wall architecture (Willats, W. G. et al. 2001a) . Table 1.1 summarizes the available anti-pectin mAbs.

Table1.1. Anti-pectin monoclonal antibodies (mAbs)

Antibody	Antigen/Epitope	Reference
JIM5	HG / Low or not methyl-esterified (up to 40%)	(Knox, J. P. 1997)
JIM7	HG / High methyl-esterified (up to 80%)	(Knox, J. P. 1997)
2F4	HG / dimerised oligogalacturonides (“egg-box”)	(Liners, F. et al. 1992)
PAM1	HG / blockwise pattern of de-esterification (30 contiguous non-esterified GalA residues)	(Willats, W. G. et al. 1999)
LM7	HG/ nonblockwise pattern of de-esterification (4 unesterified GalA residues between methyl-ester groups)	(Willats, W. G. et al. 2001b)
CCRC-R1	RG-II monomer	(Williams, M. N. et al. 1996)

Antibody	Antigen/Epitope	Reference
CCRC-M1	RG-II borate-crosslinled dimmer	(Puhlmann, J. et al. 1994)
CCRC-M2	RG-I	(Puhlmann, J. et al. 1994)
CCRC-M7	RG-I / arabinosylated galactan	(Puhlmann, J. et al. 1994)
LM5	Galactan	(Jones, L. et al. 1997)
LM6	Arabinan (may also bind to arabinogalactan proteins)	(Willats, W. G. et al. 1998)
LM8	XGA	(Willats, W. G. et al. 2004)
LM9	Feruloylated-(1→4)-ß-D-galactan	(Clausen, M. H. et al. 2004)

Immunofluorescent labeling is an excellent technique for studying distribution of structurally different domains of HG. Using this approach it was found that the degree and pattern of methyl-esterification of HG is spatially-regulated in cell walls in relation to intercellular spaces. For example, HG with a non-blockwise distribution of methyl esters (labeled by LM7) is restricted to the cell corners (regions of cell walls lining intercellular spaces) and are particularly abundant at the junction between adhered and separated primary cell walls (Willats, W. G. et al. 2000). Long non-esterified blocks of HG (labeled by PAM1) are restricted to the lining of intercellular spaces and regions of the cell wall close to the plasma membrane, other than those near intercellular spaces (Willats et al., 1999).

Spectroscopy

A wide application of spectroscopic methods has been developed for the analysis of cell wall polysaccharides (McCann et al.1992; McCann, Roberts 1994). These powerful non-invasive techniques based on vibrational spectroscopy allow for a quantitative detection of a range of functional groups, leading to a complex carbohydrate “fingerprint” of plant tissues (Chen et al., 1998). These methods present several advantages in terms of speed, ease of preparation of the plant material and high throughput, allowing analysis not only on the cellular level, but also on separate sites of the cell wall. The combination of microscopy techniques with these procedures allow for measurements on separate areas of the cell wall of an individual cell (≥ 10 um) (McCann et al. 1992). Three kinds of spectroscopy have been used for analyzing cell walls: infrared (IR), Raman and near infrared (NIR) spectroscopy, all of which are based on the

physical processes of absorption and dispersion. Fourier transformed infrared (FTIR) spectroscopy is the technique that has been most widely used. Use of the polarized infrared beam allows for a rough chemical characterization of the composition of the studied surface, also including an evaluation of cellulose and pectin orientation (Jarvis, McCann 2000).

Though the infrared spectra of polysaccharides are complex, the basic polymers of the cell wall (HG, RG-I, xyloglucan, arabinoglucuronoxylan, glucomannan and galactoglucomannan) can be identified due to absorption in the region around 1200-800 cm^{-1} (Kacurakova et al. 2000). Free carboxylic groups of uronic acids present an absorption band at 1607 cm^{-1} while their esterified modifications absorb at 1740 cm^{-1}, allowing for an estimation of the degree of esterification of HG. The compiled spectra yield a carbohydrate "fingerprint" of the cell walls, which allows for the identification of changes in the components of cell walls. As a result of this, these methods have been applied to the screening of cell wall mutants of *Arabidopsis* (Chen, L. et al. 1998, Mouille, G. et al. 2003).

1.2.3. Biosynthesis of pectin

Because of the structural complexity and diversity of pectins, a recent estimate shows at least 54 different enzyme activities (glycosyl- methyl-, acetyl- and feruloyl-transferase activity) and at least 12 different nucleotide sugar substrates are required for biosynthesis of pectin (Mohnen, D. 2008).

Localization of pectin biosynthesis

The first insights on pectin biosynthesis were gathered from immunocytochemical studies on electron microscopy sections of plant cells labeled with anti-pectin antibodies (Moore, P. J. et al. 1991). HG – like and RG-I – like epitopes were found in the cis-Golgi, with branching of RG-I taking place in the trans-Golgi, while HG becomes esterified in the medial and trans- Golgi (Lynch, M. A. and Staehelin, L. A. 1992, Staehelin, L. A. and Moore, I. 1995, Zhang, G. F. and Staehelin, L. A. 1992). The synthesized polymers are targeted to the wall by the movement of the Golgi vesicles, presumably by actin filaments (Nebenfuhr, A. et al. 1999). After insertion of HG into the primary cell wall, the degree of methyl-esterification decreases, with less methyl-esterified HG in the middle lamella. New data have also confirmed the Golgi localization of pectin biosynthesis, showing that the first enzyme described in this process

(GAUT1) is localized in the Golgi (Sterling, J. D. et al. 2006). However, it cannot be excluded that some early stages of pectin biosynthesis already take place in the endoplasmic reticulum.

Involvement of GTs in pectin biosynthesis

Because of the complex structure of pectins, it is still not clear if the synthesis occurs in a processive or distributive manner. To date no evidence for *in vitro* processivity of pectin synthesis has been reported. It is also unclear weather RG-I is synthesized separately from HG or on the same polymer chain. Structural analyses show that RG-I is covalently associated with xylogalacturonan (XGA) (Schols, H. A. et al. 1995, Zhang, Z. et al. 1996). This indicates that RG-I and HG may exist as a single polymer. For RG-II, based on cell wall extract analysis, it is likely that its synthesis occurs on existing HG since this structure is covalently linked on both ends to HG regions (Ishii, T. and Matsunaga, T. 2001).

The synthesis of HG requires at least one homogalacturonan α 1,4-galacturonidetransferase (HG-GalAT), a homogalacturonan-methyltransferase (HG-MT), and a homogalacturonan 3-O-acetyltransferase (HG-AT). So far, only one enzyme involved in HG biosynthesis has been biochemically identified – GAUT1 (Sterling, J. D. et al. 2006) (see table 1.2. for a description of known GTs). As mentioned previously, because of the complexity of RG-II structure, a large number (24) of transferase activities are required for its biosynthesis. Strong evidence has been shown for only two xylosyltransferases (RGXT1 and RGXT2) with documented *in vitro* activity and apparent involvement in the making of a RG-II side chain (Egelund, J. et al. 2006) and for a putative glucuronosyltransferase (GUT1) involved in making RG-II (Iwai, H. et al. 2002). The synthesis of RG-I however, presumably requires 30 transferase activities for all of the known structures of RG-I. So far, none of these enzyme activities have been shown *in vitro*. However, functional genomics analyses and mutant studies have identified two putative enzymes: a putative arabinosyltransferase (ARAD1) involved in making arabinan (Harholt, J. et al. 2006), and a putative xylosyltransferase (XGD1) involved in XGA synthesis (Jensen, J. K. et al. 2008). In several other cases putative enzymes involved in pectin biosynthesis have been identified, but because of the structural redundancy of different cell wall components, their specific activities are still somewhat unclear.

Table 1.2. Identified and putative pectin biosynthetic enzymes.

Enzyme	Monosaccharide transferred	Polysaccharide produced	Confirmed biochemical activity	Reference
GUT1	Glucuronic acid	RG-II	no	(Iwai, H. et al. 2002)
QUA1	Galacturonic acid	HG?	no	(Bouton, S. et al. 2002)
RGXT1 RGXT2	Xylose	RG-II	yes	(Egelund, J. et al. 2006)
ARAD1	Arabinose	RG-I	no	(Harholt, J. et al. 2006)
GAUT1	Galacturonic acid	HG	yes	(Sterling, J. D. et al. 2006)
XGD1	Xylose	XGA	no	(Jensen, J. K. et al. 2008)

Despite significant progress in understanding pectin biosynthesis achieved in the past few years, many unanswered questions remain as to how pectins are synthesized. For example, the structure of newly synthesized pectin is still unknown. It is also elusive if the interconnection of different polymers occurs during or after the insertion in the cell wall. How are the main three domains of pectin (HG, RG-I and RG-II) linked together during synthesis remains to be tested It is also unknown if variations of the degree of esterification and the pattern of esterification arise in the cell wall or partly during synthesis.

1.2.4. Modification and Degradation of the Pectin

Modification of pectin

Pectin modifying activities include pectin methylesterases (PMEs), pectin acetylesterases (PAEs) and pectin-binding peroxidases. Table 1.3 lists the presumed number of genes in *A. thaliana* for each of these enzymes. PMEs remove the methyl ester from esterified HG. They are usually developmentally and spatially regulated (Louvet, R. et al. 2006, Minic, Z. et al. 2009). There are generally three modes of action for PME: the “multiple-chain” mechanism which leads to random de-esterification, the “single-chain” mechanism, which leads to blockwise de-esterification and the “multiple attacks” mechanism, where the PME de-esterifies a fixed number of residues within an esterified block, leading to fragmented de-esterification (Grasdalen, Hans et al. 1996). Studies of PME action *in vitro* suggests that acidic PMEs (such as fungal PMEs) tend to follow a more random pattern of de-esterification, while alkaline PMEs (like those found in plants) result in blockwise de-esterification (Denes, J. M. et al. 2000, Limberg, G. et al. 2000). However, the mode of action *in vitro* may not reflect the actual action

in muro. More recently, studies have proposed possible roles of PME in systemic movement of tobacco mosaic virus in plants (Chen, M. H. and Citovsky, V. 2003) and as enhancer of RNA silencing (Dorokhov, Y. L. et al. 2006).

PAEs act in a fashion similar to PMEs in that they hydrolyze the ester bond between a glycosyl residue and an acetyl group. However, the role for these enzymes is not as firmly established as for PMEs.

Degradation of pectin

Pectin degrading enzymes can be classified into two groups: hydrolases and lyases. Hydrolases require a water molecule for their reaction, while lyases act by β-elimination, without the use of water. The structural differences between different pectic domains (RG-I, RG-II and HG) have implications for the enzymes involved in the degradation of these regions. The backbone of HG can be hydrolyzed by pectate lyases (PL), and exo- and endo-polygalacturonases (exoPG and endoPG) (Hadfield, K. A. and Bennett, A. B. 1998, Linhardt, R. J. et al. 1986). RG-I can be depolymerised by RGase and RG-I lyase (Herron, S. R. et al. 2000, Wong, D. 2008) acting on the backbone of the polymer and by β-galactanase and α-arabinanase acting on the side chains. Table 1.3 lists the pectinolytic enzymes so far found in *A. thaliana*. The distribution of these specific enzymes varies within plant tissues and they can occur in several different isoforms (Willats, W. G. et al. 2001a) .

Table1.3. Known pectinases and estimates of gene copy number in *A thaliana* (based on search at www.cazy.org).

Enzyme	*A. thaliaina* gene copy number
Pectinmethylesterase (PME)	66
Pectin acetylesterase (PAE)	11
Endopolygalacturonase (endoPG)	65
Exopolygalacturonases (exoPG)	4
Pectate lyase (PL)	27
β-Galactosidase	15
α-Arabinosidase	1
RG-I lyase	7
Pectin binding peroxidase	5

Polygalacturonases have been shown to be secreted by a wide range of pathogens (Prade, R. A. et al. 1999) and developmentally expressed in certain plant tissues (abscission zones,

pollen, flowers and fruits) (Brown, S. M. and Crouch, M. L. 1990, Kalaitzis, P. et al. 1997). These enzymes are virulence determinants crucial in the degradation of pectins by pathogens such as *Alternaria* and *Erwinia* and are the first cell wall degrading enzymes secreted upon infection (Isshiki, A. et al. 2001, Lei, S. P. et al. 1985).

Pectate lyases (PLs) break the α-(1→4) linkage between GalAs by a β elimination, causing the formation of a double bond between C-4 and C-5 atoms of one of the GalAs. Unlike PGs. which tend to have acidic pH optima, PLs seem to be most active at alkaline pH and are also calcium-dependent (Marin-Rodriguez, M. C. et al. 2002).

1.2.5. Cell and developmental biology of pectin

Pectins are highly abundant in the middle lamellae, where they function in maintaining cell adhesion (Lord, E. M. and Mollet, J. C. 2002). In addition, multiple lines of evidence indicate a role for pectins in several physiological processes: plant growth and development, morphogenesis and defense against pathogens (Ridley, B. L. et al. 2001). On the cellular level pectins are involved in defining wall structure and porosity, signaling, cell expansion and the above mentioned cell-cell adhesion (Willats, W. G. et al. 2001a) . Besides the ubiquitous functions, they also have a significant role in specific cell type activities and specific organ development, such as pollen tube growth and microsporogenesis, seed germination and hydration, leaf abscission and fruit development (Jiang, L. et al. 2005, Lacoux, J. et al. 2003, Prasanna, V. et al. 2007, Ren, C. and Kermode, A. R. 2000, Roberts, J. A. 2000, Steele, N. M. et al. 1997). The involvement of pectins and pectin modifying enzymes in these processes is further discussed in this chapter.

Microsporogenesis and pollen tube growth

During pollen development, pectin degradation in the walls of pollen mother cells needs to occur in order for the microspores to be separated. This has been shown in a series of mutants in *A. thaliana* affected in pollen separation, named QUARTET (QRT) loci, where the four products of microsporogenesis remain fused and pollen grains are released as tetrads (Rhee, S. Y. and Somerville, C. R. 1998). Two of these loci encode a pectin methylesterase (QRT1) and an endopolygalacturonase (QRT3) (Francis, K. E. et al. 2006, Rhee, S. Y. et al. 2003). These genes play a direct role in cell type-specific pectin degradation in the pollen mother cell wall

during microspore development. These mutants provide valuable genetic tools: in *A. thaliana*, tetrad analysis in *qrt* mutants has been used to map all five centromeres, to easily distinguish sporophytic from gametophytic mutations, and to accurately assess crossover interference (Preuss, D. et al. 1994).

Pollen tube cell growth occurs by tip growth (Hepler, P. K. et al. 2001). The process of tip growth has two distinct characteristic regions: the apical growing region and the distant mature region. In the apical growing region the cell wall consists of a pecto-cellulosic layer, abundant in pectin, especially highly methyl-esterified HG. The distant mature region, despite the pecto-cellulosic layer, has a callose inner layer. In this layer HG is increasingly de-esterified by the activity of pectinmethylesterases (PMEs), which play a significant role in pollen tube growth (Bosch, M. et al. 2005).

Seed imbibition and germination

The Arabidopsis seed coat epidermis undergoes a complex process of differentiation that includes the biosynthesis and secretion of large quantities of pectinaceous mucilage, cytoplasmic rearrangement, and secondary cell wall biosynthesis (Western, T. L. et al. 2004). During seed imbibition the mucilage layer in the seed coat is released from the cell walls of the epidermis when the mature seeds are exposed to water. This pectin, or mucilage, is non-essential for viability, and compared to cell walls it is structurally simple, composed of RG-I and HG (Western, T. L. et al. 2001). A mutant screen for mucilage modification revealed several loci involved in this process. MUM2 encodes the putative beta-D-galactosidase that acts on galactan side chains of RG-I and influences the hydrophilic potential of this polymer (Dean, G. H. et al. 2007). In the absence of this enzyme, the mucilage is not released upon seed imbibitions. Therefore this enzyme is required for maturation of RG-I in seed mucilage by the removal of galactose/galactan branches, resulting in increased swelling and extrusion of the mucilage during seed hydration (Dean, G. H. et al. 2007).

Cell elongation

Cell elongation is constricted by the extensibility of the cell wall; underlying this process are the mechanical properties of the cell wall that control extensibility. Recently it has been

shown that the degree of pectin methyl-esterification (DM) limits cell growth, and that a minimum level of about 60% DM is required for normal cell elongation in Arabidopsis hypocotyls (Derbyshire, P. et al. 2007). Low average levels of pectin DM are associated with reduced cell elongation, implicating PMEs, the enzymes that regulate DM, in the cell elongation process.

Fruit ripening

One of the most important processes involved in fruit ripening is the modification of pectins, which leads to loss of texture and softening of the tissue. The main enzymatic activity responsible for this process is polygalacturonase (Fischer, R. L. and Bennett, A. B. 1991). Another enzyme that is highly active during fruit ripening is PME (Prasanna, V. et al. 2007). The degree of pectin methylesterification decreases from 90% to 35% during tomato fruit ripening (Koch, J. L. and Nevins, D. J. 1989). The exact role for this modification has not yet been determined, but it is known that the activity of polygalacturonases (PGs) is higher on low-esterified pectin.

One interesting fact is the first commercially grown genetically engineered food was the tomato that produces antisense RNA interferes with the expression of and production of. endoPG (Sheehy, R. E. et al. 1988).The intended effect was to slow down the softening so that fruits could be harvested like green tomatoes without greater damage to the tomato itself, therefore increasing the shelf-life and preserving the flavor (popular name for this gemetically mosified tomato is Flavr Savr). Flavr Savr turned out to disappoint researchers in that respect, as the antisensed endoPG gene had a positive effect on shelf-life, but not on the fruit's firmness, so the tomatoes still had to be harvested like any other unmodified tomatoes.

Cell separation processes (abscission and dehiscence)

During organ abscission and dehiscence, both processes where cell separation is necessary, it is shown that the major factor involved is the breakdown of the middle lamellae caused by several pectinolytic enzymes, particularly endopolygalacturonases (endoPG) (Roberts, J. A. 2000). Increased activity of these enzymes have been reported in the abscission zones of leaves, flowers and fruit (Roberts, J. A. 2000). There is also evidence for the action of

endoPG in the floral abscission zone, dehiscence zone of siliques and anthers and during pollen development in *A. thaliana* (Rhee, S. Y. et al. 2003, Sander, L. et al. 2001) However, studies on structural changes of pectin in abscission and dehiscence zones have been very limited.

1.3. Microbial pectinases

Plant saprophytes and parasites secrete several pectin degrading enzymes, differing in substrate specificity, mode of action, and activity, in order to achieve synergy in the degradation of pectin. Fungal pectinases are classified into two mayor groups: hydrolases and lyases (Jayani, R. S. et al. 2005). The hydrolase family encodes exo- and endo- polygalacturonases, rhamnogalacturonan hydrolase and xylogalacturonan hydrolases, and the lyase family includes pectin-, pectate-, and rhamnogalacturonan lyases (Jayani, R. S. et al. 2005). Other enzymes involved in pectin degradation are referred to as accessory enzymes, and consist of xylosidases, arabinofuranosidases, endo- and exo-arabinanases, galactosidases, endo- and exogalactanases. Fungal pathogens have a suite of enzymes for degrading plant polysaccharides organized in large gene families encoding several enzymes acting on pectin (Prade, R. A. et al. 1999).

Most pectinolytic enzymes are produced from saprophytic or parasitic fungi. The members of the fungal genus *Aspergillus* are commonly used for the production of pectinases (de Vries, R. P. and Visser, J. 2001). Moreover, there are isoforms of these enzymes which recognize extended sites of different pectin structures. For example, there are endoPGs that can act on unesterified or highly esterified HG, moreover with special affinity to different patterns of methyl-esterification (Tewari, R. et al. 2005).

Microbial pectinases as a tool to study pectin structure and composition

A very powerful tool in the structural analysis of polysaccharides is the degradation into smaller oligomers that can be structurally characterized. This can be achieved by the use of endo-hydrolytic enzymes that specifically and selectively hydrolyze pectins (Bauer, S. et al. 2006). By using the enzymes to hydrolyze specific polysaccharides, it is possible to selectively investigate one type of cell wall polymer at a time. MALDI-TOF MS is a useful tool to study this type of polysaccharide digest (Daas, P. J. et al. 2000). Daas and colleges applied this methodology to study pectin methyl-esterification pattern by using pectinases (Daas, P. J. et al.

2001b). The data were used to draw conclusions about the mode of action of pectinases as well as the structure of the native pectin (Daas, P. J. et al. 2001b).

Another approach of enzymatic fingerprinting of polysaccharides is PACE (polysaccharide analysis by capillary gel electrophoresis), which yields quantitative and structural information on the original pectin chain. It was found that HG constitutes a high proportion of the cell wall material (up to 15%), the majority of which (60%-80%) was highly esterified in a blockwise fashion (Barton, C. J. et al. 2006).

1.4. Plant Innate Immunity

Plants are continually exposed to pathogens and have evolved a series of defense mechanisms. A sophisticated surveillance system acts in the plant to detect the presence of pathogens and is interconnected with the plant defense signaling pathways. This ultimately leads to the activation of defense responses, including the accumulation of secondary metabolites with anti-infective activities against bacterial and fungal pathogens (Dixon, R. A. et al. 1994). These mechanisms together comprise the so called “innate immunity” that defends plants from invading pathogens (Jones, D. A. and Takemoto, D. 2004). Recognition of the so-called pathogen-associated molecular patterns (PAMPs) that are common to different pathogens is a key event in plant innate immunity (Ingle, R. A. et al. 2006). The cell wall plays an instrumental role in survival and adaptation to stresses imposed by pathogens: an important part of the plant surveillance system resides in this compartment (Vorwerk, S. et al. 2004).

1.4.1. Pectin mediated signaling

Amongst the most abundant proteins secreted by microbial pathogens are various endoPGs (D'Ovidio, Mattei, Roberti, and Bellincampi 2004). When these enzymes degrade HG, the formation of OGAs occurs (linear molecules of 2-20 GalA residues). Accumulation of OGAs is favored when endoPGs interact with specific plant cell wall proteins (polygalacturonase-inhibiting proteins or PGIPs) (De Lorenzo G., D'Ovidio, and Cervone 2001;Di, Zhang, Xu, Cheng, and An 2006). Plant cell wall-derived OGAs trigger the typical responses elicited by PAMPs. Minute concentrations of OGAs activate the expression of defense genes via a signal transduction pathway that functions independently of the known pathways involving salicylic acid (SA), jasmonate (JA), and ethylene (ET) mediated signaling

(Stennis, Chandra, Ryan, and Low 1998;Ferrari, Galletti, Denoux, De, Ausubel, and Dewdney 2007). The first response observed after OGA elicitation is the production of reactive oxygen species (ROS), including H_2O_2 and O_2^- (Low and Merida 1996) (see fig.1.7). This rapid and transient production of large amounts of ROS, referred to as oxidative burst, is an intermediate and localized reaction of plant defense (Doke 1983; Wojtaszek 1997). Although potentially damaging, ROS is thought to have potential for being a signal in plant defense responses and has been shown to promote plant resistance to pathogens in several ways (Mullineaux et al. 2000; Neill et al. 2001, Bolwell 1999).

The OGA induced defense response is still poorly investigated, and the identification of the OGA receptor on the plasma membrane is still not reported. Wall associated kinases (WAKs) are likely to take on this role, since some studies show that these proteins interact with OGAs in a Ca^{2+}-dependent manner (Decreux and Messiaen 2005).

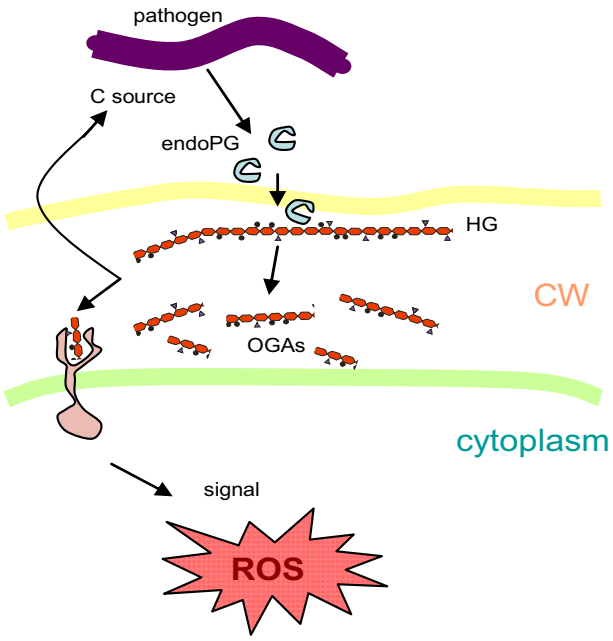

Fig1.7. Simplified view of pectin mediated signaling. Penetration of the cell wall by a pathogen results in enzyme-mediated release of oligogalacturonides (OGAs) that diffuse freely within the aqueous environment of the wall and can be used as carbon source by the pathogen (C source). It has been proposed that these OGAs also serve as signal molecules that bind to receptors and trigger defense responses. CW – cell wall, ROS – reactive oxygen species. The putative OGA receptor is depicted as a transmembrane protein.

1.5. Commercial use of pectin

Pectin is a highly valued functional food ingredient, classified as a soluble dietary fibre, and it is widely used as a gelling agent and a stabilizer. Worldwide consumption is estimated at around 45 million kilograms, with a global market value of at least 400 million € (Savary, B. J. et al. 2003). Currently citrus peel and apple pomace are the major sources of extracted pectin (Willats, W. et al. 2006). The health effects of pectin make this compound even more attractive. It is widely accepted that a high fibre diet is beneficial to health and pectin is an important soluble fibre in fruits and vegetables (Waldron, K. W. et al. 2006). There is clear evidence that pectin can lower cholesterol levels, serum glucose levels, and may also have anti-cancer activities and stimulating effect on the immune response (Inngjerdingen, K. T. et al. 2007, Jackson, C. L. et al. 2007, Kelsay, J. L. et al. 1981, Yamada, H. 1996). Pectin is also used in the production of a variety of specialty products such as adhesives and biodegradable films, foams and plasticizers, materials for drug delivery and biomedical implantation, surface modifiers for medical devices (Mohnen, D. 2008) .

Pectinases also have a high commercial value, as the removal of pectin is just as important for some applications as is the addition of pectin to others. Pectinases are widely applied in fruit juice extraction and clarification, textile processing (scouring of cotton), degumming of plant bast fibers, waste water pretreatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives and in the alcoholic beverages and food industries (Hoondal, G. S. et al. 2002).

It is also worth mentioning that pectins are a target to biofuel related research. The better understanding of pectin structure and synthesis will lead to more refined production processes and will promote efficient biofuel production from recalcitrant plant lignocellulosic biomass (Mohnen, D. 2008).

1.6. Aim of the project

Pectins form one of the most complex polysaccharide networks known in nature. Despite their complex structure and importance to the biology of the plant cell, little is known about the molecular mechanism of their biosynthesis, modification, turnover, interaction amongst themselves and with other polymers, and their physiological function. For example, which genes influence pectin structure and metabolism? How is the pectin structure-function relationship defined? What is the role of pectins in plant innate immunity?

One way to gain insight into the pectin structure-function relationship and elucidate the genetic determinants of pectin maintenance is to identify and characterize pectin mutants. One part of this project consists of establishing a pectinase-based liquid culture screen in *A .thaliana*. *A. thaliana* was chosen as a model because of the extraordinary experimental versatility and the genetic resources available for this plant species. This screen should result in the identification of mutants with altered pectin structures and the genes that were responsible for the structural alteration.

Another strategy to answer the question about gene determinants of pectin structure consists in a reverse genetic screen of putative gene families that could be involved in this process. A focused study on the putative pectin methyl transferase gene family was chosen, since this trait of pectin structure is of high importance because it is involved in many cell processes including pectin-mediated signaling.

Together these approaches should give us more insight on the contribution of pectins to the development of plants, their dynamic regulatory function and their role in plant pathogen defense.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from Sigma-Aldrich, Fluka Chemie Gmbh, and Amersham unless otherwise noted. Molecular biological reagents and kits were purchased from Amersham Bioscience, Qiagen and Invitrogen. α-amylase, pululanase, endo-polygalacturonase (endoPG) and pectate lyase (PL) were obtained from Megazyme. The purified recombinant pectin methylesterase (PME) from *A. aculeatus* was a gift from K. Schnorr (Novozymes, Denmark). All other enzymes were purchased from Roche Diagnostics and Sigma-Aldrich. The trimethylsilylation reagent (TriSil®) was purchased from Pierce (Rockford, USA). Only Mili-Q water was used in the experiments.

2.1.2 Synthetic oligonucleotides

All oligonucleotides were synthesized by Integrated DNA Technologies, USA or by MWG Biotech, Germany.

2.1.2.1 PCR primers for TAIL-PCR

Arbitrary degenerate (AD) Primer Sequences:

	Primer sequence (5'–3')	Length	Degeneracy	Tm
AD1	NGTCGASWGANAWGAA	16 bp	128-fold	46.6^{0}C
AD2	TGWGNAGSANCASAGA	16 bp	128-fold	49.2^{0}C
AD3	AGWGNAGWANCAWAGG	16 bp	128-fold	46.6^{0}C
AD6	WGTGNAGWANCANAGA	16 bp	256-fold	45.3^{0}C

W = A or T, S = G or C, N = A or T or G or C.

Left border (LB) T-DNA Primer Sequences :

	Primer sequence (5'–3')	Length	Tm
SKI1	AATTGGTAATTACTCTTTCTTTTCCTCCATATTGA	35 bp	56.8^{0}C
SKI2	ATATTGACCATCATACTCATTGCTGATCCAT	31 bp	57.9^{0}C
SKI3	TGATCCATGTAGATTTCCCGGACATGAA	28 bp	59.4^{0}C

2.1.2.2 PCR primers for genotyping T-DNA lines

T-DNA and Transposon specific primers:

Primer sequence (5'–3')

SALK_LBb1.3 (for SALK T-DNA lines): ATTTTGCCGATTTCGGAAC

SAIL_LB1 (for SAIL lines): GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

GK_LB: (for Gabi-Kat T-DNA lines): ATATTGACCATCATACTCATTGC

Spm1 (for SM transposon lines): CTTATTTCAGTAAGAGTGTGGGGTTTTGG

Ds3-1 (for GT transposon lines): ACCCGACCGGATCGTATCGGT

Genomic DNA (gDNA) specific primers:

The gDNA specific primers were designed by Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using a query sequence of 1200bp that contains the insertion with the following settings: product size of 900-1200bp, primer size of 25-27bp, Tm optima of 60^0C and GC content of 40%.

Table2.1 List of primers for all 32 pec mutants. LP – left primer, RP – right primer

pec#	gene ID	primer	primer sequence
pec100	At2g44280	pec100-1_LP	TTGACAAATGGCAAGTCTTTAATAAG
		pec100-1_RP	CAACTAATCGTTTTCAAAAGAGACAA
		pec100-2_LP	GGCATTTTAAAGAGCCTAAATAACAC
		pec100-2_RP	TCTTTTGAAAACGATTAGTTGATGAC
pec102	At3g17640	pec102-1_LP	CATGAAAATCGACCTGTAATTTCTTA
		pec102-1_RP	GGAGATTTGAGAGAGATGAGAAAGAT
		pec102-2_LP	ATGAAGAAAAATAGAGAAAAGGACCA
		pec102-2_RP	ATCTTTCTCATCTCTCTCAAATCTCC
pec104	At2g4000	pec104-1_LP	CAACGTAGAAATGTGTCTCTCGATCT
		pec104-1_RP	GGGGTTATATAAGTATCAAACGGAGA
		pec104-2_LP	ACTGTGAAAGTGAAATTGTGTTTTTC
		pec104-2_RP	TGGGGAAGAAGATCTTATATACGATC
pec105	At3g61820	pec105-1_LP	AGTTCCAACACCTAACCTCATAAAAT
		pec105-1_RP	CTTCATAGTTTCTGTATGCTTGCTTC
		pec105-2_LP	TCATTTTGCCTTTGTATACTTTTGAG
		pec105-2_RP	AGTGTTAACCGGGATCAGATAATTAC
pec106	At1g76740	pec106-1_LP	ATTTGTAGACAAAAATGAAGCAGATG
		pec106-1_RP	ATCAAAATCTTTGCGAGAAAATAGAT
		pec106-2_LP	TGGAACAGTTTGAGTTTGAAAATATC

pec#	gene ID	primer	primer sequence
		pec106-2_RP	CTCTTGAGTGTTTGTTCTTTGAGAAC
pec107	At4g31680	pec107-1_LP	TCTCTGTCTCTGTCTTTCTCTCAATC
		pec107-1_RP	ATGCTGCAAAAGTATTCAGAATTAAA
		pec107-2_LP	GACTGCAAGCTATTTGAAATACTGAC
		pec107-2_RP	ACCTAGTTGCTGTGAGATCCAATAC
pec108	At2g31800	pec108-1_LP	CCTTAGAAATCAATGGCTTTAAGAAA
		pes108-1_RP	ACACAAACATAATTTCAAAACCAAAA
		pec108-2_LP	GGAAAGAGATAAGGAAGAATGTTGAG
		pec108-2_RP	TTTCTCAACCATTCTAGCTGATTATG
pec109	At1g08290	pec109-1_LP	ATTTATCTATTGCGAATGATACACCA
		pec109-1_RP	CTCGGATATCAAAGAAGAGAATAAGG
		pec109-2_LP	GTCAAAAAGTTTTTCGAAGATTTCTC
		pec109-2_RP	CTCGGATATCAAAGAAGAGAATAAGG
pec110	At5g09360	pec110-1_LP	TTTTTCCCTCCATTTATTATTGTCTC
		pec110-1_RP	ACAGCCATATTACAATACAAAGGTGA
		pec110-2_LP	GTAATACCAACAAGATATTGACGGTG
		pec110-2_RP	TTAATGCTCGAAGAGAATCTCTTACC
pec111	At3g14225	pec111-1_LP	AAACTAGAAGGCACAATCATAAAGGT
		pec111-1_RP	TGCTTTTAGTTGACAAAACCAGTTAC
		pec111-2_LP	AAGATTGTAAGGTGCAGTGACATTAG
		pec111-2_RP	ATTGGTAATCACCAATTTGATACATG
pec112	At5g40910	pec112-1_LP	CTCACGAATTATTCACCTAGTTTTGA
		pec112-1_RP	GACGGTGTAAGATTAAGTTTGTTTGA
		pec112-2_LP	TATAGTGTCACTTTTGACCTTGCTTC
		pec112-2_RP	AAAGCTCTAGCAATGGTAGTCTTACC
pec113	At1g24320	pec113-1_LP	GGTGTGATTCTACTCCTTTCCTTAAC
		pec113-1_RP	CCTGCAACTCTATAAGTATGACTCCA
		pec113-2_LP	TAAACCTTGTTCTCTACATCGCATAC
		pec113-2_RP	AGGAAGAGAAGTCTGGACTACTTCAG
pec114	At1g58370	pec114-1_LP	GTAGATAGCTTGGCTTGTTTACCAAT
		pec114-1_RP	CTAGGAATTCTCGGTTTACCAATATG
		pec114-2_LP	CTCAATAGCTTAACTGTAAAACACGC
		pec114-2_RP	CTTGGTAGAATGATCCATGTAACATC
pec115	At1g11545	pec115-1_LP	AAAAATGTAAGAATACCGGATTATGC
		pec115-1_RP	TCGTTATCTAAGGAAAGATTCCAGAT
		pec115-3_LP	GACGAGATAGATTTCGAATTTCTAGG
		pec115-3_RP	AAAAGCAGTTATGAAATATAGCCGAC
pec116	At1g08010	pec116-1_LP	CAAAACTTAAAAGTCCCAAAACAAGT
		pec116-1_RP	ATGAATTAACTCGCTTCTTGAAGTCT
		pec116-2_LP	TTGCAAGAAGAGGTTTAATGTTACAG

pec#	gene ID	primer	primer sequence
		pec116-2_RP	ATTAGGAATACCCTCTACATGAATGC
pec117	At1g05240	pec117-1_LP	GAGAAAAACGTGTAGACTCAAAATCA
		pec117-1_RP	CTTTTGTAATGATTCATGTAGGGATG
		pec117-2_LP	TGGAAATATCTTGAAAAAGGATTTTC
		pec117-2_RP	CAGTTTTGGAATGACAAAATCACTAG
pec118	At5G09280	pec118-1_LP	GGGGTTCTTATACTGTTGTCTATGGT
		pec118-1_RP	CCAATAACTATTACCAAGGATGGACT
		pec118-3_LP	TAAGATCCATGATTGTAAAGCTCATC
		pec118-3_RP	ACAATCAGAACCATTGTCTCTGTTAG
pec119	At1g03700	pec119-1_LP	GAATACGTAGAGAACCAATGCAACTA
		pec119-1_RP	GCTTGAAAAACGTCTATGGTTTTACT
		pec119-2_LP	CCATCCAACACTTGAATAACATATTC
		pec119-2_RP	AATAAAAATCCACGTGTCATAATTTC
pec120	AT5G01030	pec120-1_LP	GTTTAGACTCTACCAACGGGAGATAG
		pec120-1_RP	CTATGAGATTTGATGGTTCAGTTTGT
		pec120-2_LP	GATTGTGAGCTGAAAAATAGCTAGG
		pec120-2_RP	GGTAGAGATCACAAAGGAGTAGAACC
pec121	At1g09950	pec121-1_LP	TGTATGTGAAAACGGTATCTAAATCG
		pec121-1_RP	AAATCCTACGTAGCTTATGATGTGGT
		pec121-2_LP	TTCAGATGTTATTTTAGGTCATTCCA
		pec121-2_RP	AATCTCGCATAACTTCTCTCTCTTTC
pec122	AT4G35830	pec122-1_LP	CAACCTTAGATACTGCAAAGTGACAT
		pec122-1_RP	ACATAATGATGTTTCTGATTTCCTGA
		pec122-2_LP	GATCATTGAATATTTTTGGTGTCTTG
		pec122-2_RP	CATTTCTCTCATAAAACTCTCACCAA
pec123	At5g40470	pec123-1_LP	TTAATCTTAGGCTTTAGCACAAACAC
		pec123-1_RP	TAGATAGAGAGGGAAGAGAGACAAGG
		pec123-2_LP	AAAAATGGAAGAAATTTTATTCGATG
		pec123-2_RP	AAGTCTTCAACTTCACACAGTTTTTG
pec124	AT1G50110	pec124-1_LP	GGTTTGTTTGTTGTCAGTCACACTAT
		pec124-1-RP	CGACAAATTGTTCTAGAGTAGGAGGT
		pec124-2_LP	ACAAAGAACGGCCACTTTAGC
		pec124-2_RP	TACCCATTTCTTGTTGGCAAG
pec125	At4g01400	pec125-1_LP	TGTTATTAAGTATCGTCATGCCAAA
		pec125-1_RP	AGTGAGAGATAAGTTTGCTCGGTTA
		pec125-2_LP	ATTGACTTATTTGGTGACAACTATGG
		pec125-2_RP	ATCTTATAAGAATCCACATCAGGGAC
pec126	At5g25150	pec126-1_LP	AGTACCACATTCCCACATATAATTCC
		pec126-1_RP	AACCTTTCCTCTAACACCTAGGATTC
		pec126-2_LP	CACAGAATCAATTGACGAGTAAAAAC
		pec126-2_RP	TGACGAGATGATTCTAATTTTGTAGC

pec#	gene ID	primer	primer sequence
pec127	At4g35170	pec127-1_LP	GTTGAGAGAACTCTCCCATCTATTTT
		pec127-1_RP	AACACAGACGTAGACAAGATATCGAC
		pec127-2_LP	CAATAAAATGTGAAATGGTTTACCTG
		pec127-2_RP	CCACATTTGACCTAATATCACAAAAC
pec128	At5g14890	pec128-1_LP	TGTTTCTTCTAATGTATCTCCCCTTT
		pec128-1_RP	AAGATAACCGAGAGAAGAACGAGAT
		pec128-2_LP	CCAATTTATCAACGGTTCTGATTC
		pec128-2_RP	AAGAGTAAGAGACATGTAGCAACACC
pec129	AT1G26240	pec129-1_LP	TTTGTAAAATCATATGACCCAAAGAG
		pec129-1_RP	TGAAATATGGATGTTATGGTAATTCG
		pec129-2_LP	GAGCTATAGATATGTGTAGGAGGTTTG
		pec129-2_RP	AAGTGGCGTCCTTAAATTAGACTAT
pec130	At3g59830	pec130-1_LP	ACTCTTTGACTTTGTTCTGATTGTTG
		pec130-1_RP	AAGGTTTGTTGATTTTCCTAATTGAC
		pec130-2_LP	GAGATTAGAAAATAGTGATTACCCGC
		pec130-2_RP	TGCGTACTTACTTTTGGATTACACTC
pec131	At5g18990	pec131-1_LP	TACTTTCCATAGAAAAGGTTCCAATC
		pec131-1_RP	TTTGTTGTTTTTGTCTACCTCATCTC
		pec131-2_LP	AAAAGAAGGAATCATCATAGGAAATG
		pec131-2_RP	ACGAATAAAAAGTCTCGACCACTAAC
pec132	At2g39440	pec132-1_LP	CACATAGATAATTTGACCCAAAACAG
		pec132-1_RP	TATGTTTTGGTTATTGACAATTCGTT
		pec132-2_LP	GTACAGTAGAGAACCTGGTGAATTTG
		pec132-2_RP	TTTACTCCATCGATTTCAACATCTAC
pec133	At1g15980	pec133-1_LP	TATCTACCTGAACACCAGGATACCTT
		pec133-1_RP	TTCTGCTTTTTCTGTTCTTTTGTTC
		pec133-2_LP	CGGTCAATCTCAAGAAGAAGAATAAT
		pec133-2_RP	TATACCTGAAACCATCAACAGTCAAT
pec134	AT2G46110	pec134-1_LP	AAAAGCTATAGTTGAAGCAGGAATTG
		pec134-1_RP	CCGTAAATATATGAAACTTGCTTCTG
		pec134-2_LP	AAGAAGATCATGGTAAACTAGCACCT
		pec134-2_RP	CTACGAGTCAAGCTCTAGTCAGGTAA

2.1.2.3 RT-PCR primers

Exon spanning primers were designed for amplification of *PEC100* (At2g44280) and the PTB housekeeping gene (AT3g01150) cDNA.

housekeeping gene (PTB)		Tm
PTB-RT_LP	GCGAATGTCTTATTCAGCTCATACTGATC	60.2
PTB-RT_RP	CATTGCTGCTGTTGGTATATCAGAGT	58.6

Pec100 – At2g442b0		
pec100mRNA_RT_LP_912	TGTATTTGGTGTTAGCGAGGCCGTG	61.2
pec100mRNA_RT_RP_1046	CTCAGCCTTGGTTCCTTTGTCCCC	61.9

2.1.2.4 PCR primers for cloning

For cloning of the promoter region of *PEC100* (from the stop codon of At2g44290 to the start codon of At3g44280) the following primers were used:

At2g44280 promoter		Tm
Promoter_pec100_LP	TGAGCTCTGCTTAACGAGAAACTTGC	59.8
Promoter_pec100_RP	AGTTCGTTGAGTCTAATGGTTTGGTT	60.1

2.1.3 Plasmids

Gateway vectors were used in this study. Entry vector pCR8/GW/TOPO was obtained from Invitrogen. Entry clone of the full length cDNA of At2g44280 (pENTR221-At2g44280) was obtained from TAIR (www.arabidopsis.org) and used for constructing an N-terminal GFP fusion plasmid, using the vector pMDC83 (Curtis and Grossniklaus, 2003). For the promoter::GUS construct pMDC164 was used (Curtis and Grossniklaus, 2003). The pMDC plasmid series were obtained from the TAIR. The ST-YFP marker control vector for Golgi vocalization (pVKH18En6::ST-YFP) was a gift from Prof. Brandizzi (see Brandizzi et al., 2002).

2.1.4 Monoclonal antibodies

All monoclonal antibodies were obtained from Plant Probes, Leeds, UK. The secondary antibody used for all of them was Anti-Rat IgG (whole molecule) FTIC conjugate, Sigma-Aldrich.

Antibody	Antigen/Epitope	Reference
JIM5	HG / Low or not methyl-esterified	(Knox, J. P. 1997)
JIM7	HG / High methyl-esterified	(Knox, J. P. 1997)
LM7	HG/ nonblockwise pattern of de-esterification	(Willats, W. G. et al. 2001c)
LM5	Galactan	(Jones, L. et al. 1997)
LM6	Arabinan	(Willats, W. G. et al. 1998)

2.1.5 Bacterial and fungal strains

Escherichia coli strains:

Xl-1 Blue Stratagene, La Jolla, CA, USA (Bullock *et al.*, 1987),

TOP10 Invitrogen, Karlsruhe, Germany, transformation efficiency 10^9 cfu/ug

Agrobacterium tumefaciens strains:

GV2260 Deblaere *et al.* (1985)

GV3101 Koncz and Schell (1986)

Alternaria brassicicola strain:
ATCC 90305 wt

2.1.6 Plant material

Arabidopsis thaliana:

Ecotypes: Columbia-0 (Col-0) and C24 were obtained from the ABRC stock center at Ohio State University.

EMS mutants:

EMS-mutagenized M_2 seeds of Arabidopsis accession Col-0 were obtained from Prof. Thomas Altmann. In brief, Col-0 seeds were mutagenized by treatment with 0.13% to 0.25% (w/v) EMS for 12 h. The M_2 seeds were bulked from pools of 600 to 1,600 M_1 plants, and resulting in 391 pools. These pools were bulked in superpools, each containing of 5 pools. This was done to maximize the likelihood that mutants would represent independent selective events rather than siblings from a single mutagenic event. The M2 plants contained a statistical average of 8 mutations per genome (pers. comm. T.Altmann).

T-DNA insertion lines:

The T-DNA activation tagged mutant seed collection used in the primary screen was provided by W.Scheible and C.Somerville, TAIR stock# Cs31100 (Weigel, D. et al. 2000). The populations used in the screen had a Col-0 background and were transformed with the pSKI15 vector. The pSKI15 vector contains multimerized transcriptional enhancers from CaMV35S which allow for induction not only of loss-of-function, but also of gain-of-function mutations. The T-DNA also carries a phosphonothricin (BASTA) resistance gene (BAR driven by 5' MAS promoter) which enables selection of transformed plants on BASTA and a pBsKS+ sequence that has a bacterial origin of replication (ori) and an ampicillin resistance ORF (amp) which allows plasmid rescue in Escherichia coli. Specific T-DNA insertion lines were obtained from

the ABRC stock center at Ohio State University, the GABI-Kat, the Genoplante FST/FLAGdb, and the University of Wisconsin Knockout facility.

Recombinant inbred lines (RILs):

The RILs derived from a cross between the two *A. thaliana* ecotypes Col-0 x Ca4 cross were obtained from Prof. Thomas Altmann (Torjek et al., 2006). In brief, F_2 plants were propagated by self-pollination using the single-seed descent method to the F_7 generation, where genotyping using 110 genetic markers and bulk amplification was performed. The final population consisted of 209 Col-0 × C24 RILs.

Nicotiana tabacum (Common tobacco)*:*

Cultivar *Petit Havana* SR1 was used for transient expression and protein localization studies.

2.1.7 Growth Conditions

Bacterial growth conditions:

E. coli and *A. tumefaciens* were cultivated in LB (Luria Bertani) or YEB (Yeast Extract Broth), as described by Sambrook et al. (1989).

Fungal growth conditions and inoculation of plants:

Alternaria brassicicola spores were grown on V-8 agar (160 ml V-8 juice, 1 g calcium carbonate, and 14 g agar per 1 liter media). Seven- to 14-day-old cultures, grown at room temperature on a 24-hr light regime, were used for inoculation. *A. brassicicola* spores (2x 10^5 spores/ml in water with 0.05 % Tween-20) were spotted as 5ul drops onto intact plants with a pipette. To maintain a humid environment conducive to fungal infection, plants were covered for 24 hr with plastic wrap, which were then slit to allow gradual equilibration (about 36 hr) with the outside environment. Symptoms were recorded about 60 hr after inoculation, when plastic wraps were removed.

Plant growth conditions:

Seeds of *A. thaliana* accessions Columbia-0 (Col-0), and the appropriate mutant lines were surface-sterilized. The seeds were washed with 70% ethanol, incubated with bleach solution containing 12% NaHClO for 15 min and washed with water. Sterilized seeds were germinated on half-concentrated Murashige and Skoog medium (pH 5.6), supplemented with 1% sucrose, and solidified with 0.7% agar under a 16-h day (140 µmol·m−2·s−1, 22 °C)/8-h

night (22 °C) regime. After 2 wk, plants were transferred to standard soil and further grown in a growth chamber under a long-day light regime (16 h of fluorescent light [120 μmol·m−2·s−1] at 20 °C and 60% relative humidity/8 h of dark at 16 °C and 75% relative humidity).

Plant liquid culture conditions:

A. thaliana seeds were surface sterilized with bleach solution (10%). About 50-100 sterilized seeds per culture were grown under sterile, long day conditions (16h light, 8h dark) at 22°C, shaking at 80 rpm in 20ml MS (Murashige and Skoog, 1962) liquid medium containing 0.5% sucrose. For the mutant screen 0.25U/ml of sterile filtered endoPG and PME (provided by Kirk Schnorr, Novozyme) or PL were added to the cultures. After 14d of growth the seedlings were examined visually and the phenotypes were compared to wild type cultures grown under the same conditions.

2.2 Methods

2.2.1 Molecular Biology Methods

2.2.1.1 Standard molecular biology techniques

Standard molecular biology techniques such as cloning, digestion with restriction endonucleases, separation of DNA fragments by agarose gel electrophoresis and preparation of common solutions and buffers were performed as described in Sambrook et al. (1989).

2.2.1.2 DNA isolation of plant material

CTAB extraction

Plant genomic DNA was extracted using a modified version of the protocol of Murray and Thompson (1980). Plant material (~300 mg) was grinded in a 1.5 mL reaction tubes. The material was incubated in 300 µL extraction buffer (2% (w/v) cetyl-trimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-Cl pH 8.0) at 65°C for at least 10 min. The insoluble material was removed by centrifugation (10 min, 14.000rpm) and the supernatant was extracted with 300 µL chloroform. The aqueous phase was then mixed with 300 µL isopropanol and DNA was precipitated and collected by centrifugation at 20,000 x g. Finally, the DNA was washed once with 70% EtOH and after air-drying re-suspended in 50 µL H2O containing 0.1 µg of RNAseA.

One-step DNA extraction

Genomic DNA was extracted using a modified version of the protocol of Edwards et al. (1991). Plant material (3-5mg) was grinded with extraction buffer (1/10 dillution of Edwards solution in TE) with a plastic rod. 1ul of this solution was used in the following PCR reactions (total of 20 ul reaction volume) (Kasajima I et al., 2004).

2.2.1.3 RNA isolation of plant material

Total RNA was isolated from plant material using the Qiagen RNA extraction kit as described by the manufactor.

2.2.1.4 cDNA synthesis

Extracted RNA (2-4 μg) was treated with one unit of RNAse free DNAse (Roche) in digestion buffer (10 mM Tris-HCl, 25 mM MgCl2, 5 mM CaCl2, pH 7.5) for 15 min @ 37^{o}C. After heat denaturation of the DNA for 10 minutes at 70 °C first strand cDNA was synthesized using SuperScript II Reverse Transcriptase and Oligo(dT)12-18 primers (Invitrogen) according to the manufacturers manual. The cDNA was subsequently used as a template in a PCR to determine the presence of gene specific transcripts.

2.2.1.5 Polymerase Chain Reaction (PCR)

Selective amplification of DNA fragments was performed using the polymerase chain reaction facilitating a thermocycler. For diagnostic purposes, Taq DNA polymerase (Invitrogen) and for cloning purposes Pfu DNA polymerase was used (Stratagene).

2.2.1.6 Modified thermal asymmetric interlaced (mTAIL) PCR

For high throughput identification of T-DNA insertion loci in selected mutants, modified thermal asymmetric interlaced (mTAIL) PCR (Sessions, A. et al. 2002) was adapted. The methods involves the amplification of a short flanking sequence of the inserted T-DNA using a sequence of two interlaced PCR's with mix of arbitrary degenerated (AD) primers and nested T-DNA border specific primers. The reactions were performed as descriebed in Sessions, A. et al. 2002. The PCR reaction was cleaned up from excess of nucleotides and primers using exonucleaaseI (ExoI) and shrimp alkaline phosphatase (SAP). To this mixture sequencing

primer was added (SKI3) and sequenced as described in 2.2.1.10. The resulting sequences were aligned with the *A.thaliana* (Col-0) genome and the approximate location of the T-DNA insertion in the respective mutants was mapped.

2.2.1.7 Confirmation of T-DNA insertion site

The T-DNA insertion lines obtained from the screen and the additional alleles from various stock centers were screened for the appropriate insert using the required T-DNA left border primer and a gene-specific primer. Gene-specific flanking primers were used to confirm homozygosity. Homozygous insertion lines were evaluated for phenotypic alterations in liquid culture with presence of pectinases as described in 2.1.7.

2.2.1.8 Sequencing

The PCR reactions were prepared for sequencing by incubation with Exopolimerase I (ExoI) and Shrimp Alkaline Phosphatase (SAP), after which the primer necessary for sequencing was added to a final concentration of 25 uM. The DNA sequencing was performed by the Genomics Core facility at MSU (http://genomics.msu.edu/).

2.2.1.9 Transcript analyses by RT-PCR

The reaction mix contained 2µl template cDNA, 0.2µM of the transcript specific primers and JumpStart REDTaq ReadyMix (Sigma) in a total volume of 20µl. As a control a second reaction was set-up to amplify a fragment of the PTB (At3g01150) transcript from the same template cDNA as a reference gene. The second reaction mix contained 2µl template cDNA, 0.2µM of the primers PTB-RT_LP and PTB-RT_RP (see 2.1.2.3.) and JumpStart REDTaq ReadyMix (Sigma) in a total volume of 20µl. Both reactions were performed at 95°C, 5min followed by 35 cycles of 95°C, 30sec; 56°C, 1min 30sec; 72°C, 2min and a final elongation for 5min at 72°C. The PCR products were separated on a 1% agarose gel stained with 0.005% ethidium bromide.

2.2.1.10 Transformation and cultivation of bacteria

Chemically competent *E.coli* cells were transformed by heat-shock as described by Hanahan (1983). After transformation, cells were grown at 37°C on LB medium supplemented by selective antibiotic as described by Sambrook et al. (1989). Competent *A. tumefaciens* cells

were prepared according to Höfgen and Willmitzer (1990) and transformed by electroporation according to Miller et al. (1988). The cells were grown at 28°C on YEB medium supplemented with selective antibiotic according to Vervliet et al. (1975).

2.2.1.11 Plant Transformation

A colony of transformed *A. tumefaciens* was inoculated and a pre-culture of 4 ml YEB, pH 7.0 (Bacto-tryptone (5 g/l), Bacto-yeast-extract (1 g/l), Bacto-peptone (1 g/l), sucrose (5 g/l) in 1 l H2O) supplied with rifampicin (25 µg/ml) and the specific selection for the vector was prepared. The mixture was incubated at 28^0C for two days. 2 ml of the preculture were further inoculated in 400 ml YEB selective medium and again incubated at 280C overnight. The bacterial cells were harvested by centrifugation at 8000 rpm for 10 min., at 40C and resuspend in 200 ml infiltration medium (sucrose (50 g/l), MS-Medium (2.2 g/l), MES (0.05 g/l), 10 µl benzamine-aminopurine in H2O, pH 5.7).

Transformation of Arabidopsis by the floral dip method:

The bacterial solution (200 ml) was added to 1 ml Silwet L-77. The plants inflorescences were dipped for 10 sec. in this solution and dried overnight (Clough and Bent, 1998). Seeds from plants were harvested and subjected to antibiotic selection on plates. Seeds were surface sterilized and sown onto 1% agar containing MS medium and kanamycin monosulphate at a concentration of 50 µg ml-1 (Melford Laboratories Ltd.), or hygromycin B at a concentration of 15 µg ml-1 (Melford Laboratories Ltd. #H0125). Seeds were then stratified for 2 d in the dark at 4°C. After stratification seeds were transferred to a growth chamber and incubated for 4–6 h at 22°C in continuous white light (120 µmol m-2 s1) in order to stimulate germination. The plates were then wrapped in aluminum foil and incubated for 2 d at 22°C. The foil was removed and seedlings were incubated for 24-48 h at 22°C in continuous white light (120 µmol m-2 s-1).

Transient expression in tobacco:

Nicotiana tabacum (cv. *Petit Havana*) plants were transiently transformed with *Agrobacterium tumefaciens*, as described in (Batoko et al., 2000). The bacterial optical density (OD600) used for plant transformation was 0.05.

2.2.2 Biochemical methods

2.2.2.1 Determination of enzyme activity

The enzymatic activity of endoPG was assayed by incubating 10 µg of total protein in ammonium formate buffer (50 mM, pH 5.0) with 50 µL of 0.5% commercial methylesterified HG for different times at 37°C in a total volume of 300 µL for incubation. The resulting reducing ends were determined with the PAHBAH (p-hydroxybenzoic acid hydrazide) – assay as described by Lever et al. (1973). One unit was defined as the amount of enzyme that released 1 mol of reducing groups per second.

The PME activity was quantified by spectrophotometric determination of methanol released from the digestion reaction as described by P. J. Wood and I. R. Siddiqui (1971). Purified protein (2 µl enzyme sample) was incubated with 4 µl of 20 mg/ml methyl-esterified homogalacturonan (DM 93% Sigma) in the presence of alcohol oxidase in sodium phosphate buffer (50 mM, pH 7.5) for 30 min. The samples were developed by incubation with a 100 µl of coloring reagent (20 µl acetyl acetone, 28 µl acetic acid, and 1.54 g ammonium acetate in 10 ml of water). Subsequently the solution was incubated at 68^0C for 10 min and absorbance was read at λ = 412 nm (UV spectrophotometer). Methanol (0.1–6 µM) was used for calibration. One unit was defined as the amount of enzyme that released 1 mol of methanol per second.

The enzymatic activity of PL was determined measuring the increase of reducing sugars released from homogalacturonan (HG) (Miller, 1959). A 50 µl aliquot of suitably diluted enzyme solution was incubated in the presence of 450 µl of 0.25% (w/v) homogalacturonan (DE 0%, Sigma) in 50 mM Tris/HCl buffer (pH 8.0) for 5 min. The reducing sugars were assayed with addition of 750 µl dinitrosalicylic acid and subsequently the solution was boiled for 5 min, cooled on ice, centrifuged and absorbance was read at λ = 540 nm (UV spectrophotometer). D-Galacturonic acid (Sigma, 0.5–3 mg/ml) was used for calibration. One unit was defined as the amount of enzyme that released 1 mol of reducing groups per second.

2.2.2.2 Detection of reactive oxygen species (ROS)

Reactive oxygen species released by leaf tissue was assayed by H_2O_2-dependent luminescence of luminol (Keppler et al., 1989). Leaves of *A. thaliana* plants were cut into approximately 1 mm slices and floated overnight on water. Slices were transferred to assay tubes (five slices corresponding to approximately 10 mg fresh weight) containing 0.1 ml of H2O

supplied with 20 uM luminol and 1 ug horseradish peroxidase (Fluka). Luminescence was measured in a luminometer (LKB Wallac) for 30 min after the addition of the test solutions.

2.2.3 Analytical and preparation methods

2.2.3.1 Preparation of crude plant cell wall material (Alcohol insoluble residue)

Plant material was harvested, pooled and frozen in liquid nitrogen. The frozen material was grounded using a beat mill (Retch) in 2 mL Eppendorf reaction tubes and immediately thrown in 70% ethanol, centrifuged for 10min at 14krpm and the ethanol was remover. The sample was further purified by additional 2-3 washing steps with 70% ethanol and subsequent wash with methanol/chloroform [1:1, (v/v)]. The remaining pellet was washed with acetone and dried over night. For light grown plant material, the cell wall material was digested with α-amylase (Sigma) by applying 50U of this enzyme per 10mg of cell wall material in 1 ml of 0.1M KPO4 buffer, pH 7.0 overnight at 30^{o}C. Afterwards the pellet was extensively washed with 0.1M KPO4 and dried again in acetone.

2.2.3.2 FTIR Spectroscopy

The prepared cell wall material (2.2.3.1.) was first dehydrated with several acetone washes. 100 spectra from each sample were collected on a Thermo-Nicolet Nexus 470 spectrometer over the range 4000 to 400 cm−1. For each spectrum, 32 scans were co-added at a resolution of 4 cm−1 for Fourier transform processing and absorbance spectrum calculation using Omnic software (Thermo Nicolet). Spectra were corrected for background by automatic subtraction and saved as JCAMP.dx format for further analysis. Using Win-Das software (John Wiley & Sons), spectra were baseline-corrected, area-normalized, and analyzed using principal components analysis covariance matrix method (Kemsley, 1998).

2.2.3.3 Sequential digest of cell walls

Aliquots (10 mg) of alcohol insoluble residue (AIR, 2.2.3.1) were sequentially extracted with 10 mL 1M KOH (Potassium hydroxide) containing 0.1% NaBH4 (Sodium borohydrate) for 24 h continuous stirring. The residual cell wall material was suspended in 10 mL 4M KOH containing 0.1% NaBH4 (24h continuous stirring) to produce extracts enriched in loosely bound and tightly bound matrix glycans, respectively. Both extracts were neutralized with concentrated

acidic acid, extensively dialyzed against double distilled water in Spectra Por ® dialysing tubes (Spectrumlabs, Breda, Netherlands) with a pore size of 3.500 Da and concentrated using a vacuum rotation evaporator to a final volume of 2 mL.

2.2.3.4 Enzymatic extraction of pectins from plant cell wall material

Pectic fractions were isolated from cell wall material (AIR, 2.2.3.1) by incubating approximately 2 mg of AIR with 8.5 U of endopolygalacturonase (Sigma, Taufkirchen, Germany) and 4.5 U pectinmethylesterase (Novozyme) in a total reaction volume of 1 mL 0.1 M NH4COOH (pH 4.7) at 37°C overnight. The mix was centrifuged for 10 min at 14.000 rpm and the supernatant was taken of for further analysis.

2.2.3.5 Size exclusion chromatography of polysaccharide containing samples

The molecular size distribution of the extracted cell wall fractions was evaluated using size exclusion chromatography (SEC). Samples (1mL) were loaded on a Superdex peptide column (Amersham Pharmacia Biotech, Piscataway, USA) connected to a fast protein liquid chromatography (FPLC) system (Biologic Duo Flow, Bio-RAD). Ammonium formate (50mM, pH 5.2) was used as running buffer and fractions of 0.5 mL/min were collected for further analysis of the sugar content over a running time of 60 min. The FPLC system was coupled to a refractive index detector (RI-Detector, RI-71).

2.2.3.6 Neutral monosaccharide composition by alditol acetate (AA) derivatization using GC-MS

Alditol acetate derivatives of neutral monosaccharides were produced from acid-hydrolyzed cell wall materials and analyzed by GC/MS (York et al. 1985). About 1mg of cell wall material and 10µg inositol as an internal standard were hydrolyzed in 2M trifluoroacetic acid. After incubation for 90min. at 121°C, the samples were cooled down and blown until dryness under a constant stream of air. The hydrolyzed samples were re-suspended in 300µl 2-propanol and evaporated, followed by reduction in excess of NaBH4 (200µl of a 10mg/ml in 1M NH4OH solution) for 1h at 40^0C. The reduction reaction was neutralized with glacial acetic acid (150µl) and dried with 9:1 methanol:acetic acid mix (3x200µl) and washed with 100% methanol (4x200µl). After reduction, the samples were acetylated by applying 50µl acetic anhydride and 50µl pyridine for 20min. at 121°C. The samples were allowed to cool down and all solvents

were evaporated under a gentle stream of air followed by two evaporations with 200µl toluene. The samples were re-suspended in 4ml water and 1ml methylenechloride, vortexed intensely and centrifuged for 2min. at 2000rpm. The methylenechloride phase containing the alditol acetate derivatives of the neutral sugars was transferred, evaporated under a gentle stream of air, re-suspended in acetone and analyzed on an Agilent 6890 GC system equipped with a 5975B inert XL MSD and a Supelco SP-2380 fused silica capillary column (30m x 0.25mm i.d. x 20µm film thickness). The injector was operated at 250°C. The transfer line to the MSD was set at 280°C. The GC was operated with temperature programming (160–245°C at 20°C/min, 245–270°C at 50°C/min). EI mass spectra were obtained from m/z 50–650 in the total ion-monitoring mode using a source temperature of 230°C, a quadrupole temperature of 150°C, a filament emission current of 34.6 µA, and an ionization voltage of 70 eV.

2.2.3.7 Neutral and acidic monosaccharide composition by trimethylsilyl (TMS) derivatization using GC-MS

Approximately 1 mg of dry cell wall material (AIR, 2.2.3.1) was subjected to methanolysis for 16 h at 80°C in 1 M methanolic HCl (0.5 ml). The mixture was evaporated by dry air and treated with Pierce TriSil® reagent (0.3 ml) for 20 min at 80°C. The samples were dried under a stream of air and the resulting residue was extracted with hexane (1ml). The hexane was concentrated to 50 µl and 2 µl used for GC-MS analysis. The silylated sugars were analyzed by GC-MS (York et al., 1985). The GC-MS was equipped with a capillary split/splitless inlet and a DB-1 fused-silica capillary column (30 m×0.25 µm i.d., 0.25 µm film thickness, J&W Scientific). The outlet of the column was connected to the mass detector by a deactivated fused-silica column. Helium (flow rate of 2 ml/min, average velocity 58 cm/s) was the carrier gas. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector was operated at 250°C. The transfer line to the MSD was set at 280°C. The GC was operated with temperature programming (120–145°C at 1°C/min, 145–180°C at 0.9°C/min, and 180–230°C at 50°C/min). EI mass spectra were obtained from m/z 50–650 in the total ion-monitoring mode using a source temperature of 230°C, a quadrupole temperature of 106°C, a filament emission current of 34.6 µA, and an ionization voltage of 70 eV.

2.2.3.8 Uronic acid content

Uronic acids were quantified using a modified protocol of Blumenkranz and Asboe-Hansen (1973). The acid - hydrolyzed cell wall material (2.2.3.6) was suspended in 100 µL of water and 600 µL of concentrated sulfuric acid containing 0.125 M Na2B4O7 were added. The solution was boiled for 5 min. After cooling 20 µL of biphenyl solution [0.15% m-hydroxybiphenyl in 0.5% sodiumhydroxide (NaOH)] were added. The mixture was vortex and the absorption was recorded at a wavelength of 520 nm on a Spectra Max Plus ELISA reader (Molecular Devices, Munich, Germany). The resulting data were compared to a standard curve of galacturonic acid.

2.2.3.9 Methyl-ester content

The degree of methylesterification was determined after saponification of cell-wall material (2 mg) with 0.5 m NaOH for 1 h at room temperature, during which time methanol is released. The resulting methanol concentration in the supernatant was determined spectrophotometrically as described in Klavons, J.A. and Bennett, A.D. (1986).

2.2.3.10 Hexose content

The hexose content of a sample was determined using a modified protocol of Dische (1964). Briefly, monosaccharides were dissolved in 250 µL of water and 500 µL of anthrone reagent (0.2% (w/v) anthrone in conc. H2SO4) was added. The mixture was vortexed and incubated for 5 min in a boiling bath. The absorbance of the reaction mixture was detected at a wavelength of 620 nm using a Spectra Max Plus ELISA reader (Molecular Devices, Munich, Germany).

2.2.3.11 Oligosaccharide mass profiling (OLIMP) by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) mass spectrometry

The supernatant (1 µL) of an endoPG/PME digest (2.2.3.4.) was spotted onto a MALDI target plate that contained 1 µL of vacuum dried matrix - crystallized 2,5-dihydroxybenzoic acid (DHB). Mass spectra were obtained using a Voyager DE-Pro MALDI-TOF MS (Applied Biosystems, Langen, Germany) in positive ion mode with an accelerating voltage of 20,000 V and a delay time of 350 ns. The recorded spectra were analyzed by calculating the relative area of the ion signals of each individual spectrum and a pair wise comparison was performed of

each individual signal corresponding to different oligogalacturonides and statistical assessment was done by a two tailed Student's t-test.

2.2.4 Microscopy and Histochemistry

2.2.4.1 Embedding and sectioning of plant tissue

Plant tissues (such as hypocotyls, stems, leaves) were fixed for 3 hours in 50 mM potassium phosphate buffer [pH6.9] containing 2.5% [w/v] glutaraldehyde. The subsequent fixation, embedding and sectioning procedure was performed as described by Freshour et al. (1996).

2.2.4.2 Antibody labeling of plant cell wall polysaccharides

The Antibody labeling was performed using the protocol of Freshour et al. (1996). The labeled sections were visualized using the fluorescence stereomicroscope Leica MZ FZ III (Leica) connected to a color view soft imaging system (Olympus, Hamburg, Germany).

2.2.4.3 UV fluorescence microscopy for phenolic compounds in the cell wall

For UV fluorescence microscopy, A. thaliana seedlings grown in liquid culture were mounted on glass slides in 0.1 M ammonium hydroxide (pH 9.8). Slides were observed using a Fluorescence Microscope (HBO 100 Hg vapor bulb, Zeiss, Jena, Germany) with a 365- ± 12.5-nm exciter and a 450-nm long-pass filter, using the 10× and 20× objectives. Wall-bound phenolic compounds fluoresce at high pH (Rudall and Caddick, 1994).

2.2.4.4 Analysis of β-glucuronidase (GUS) expression

Transformed *A. thaliana* plants carrying the *promoter::gusA* reporter gene constructs (2.2.1.11.) were tested for GUS activity, using a modified protocol from Jefferson et al. (1987). Plant material was incubated in GUS staining buffer (50 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.1% X-gluc (w/v)), and incubated at 37°C for 16 h. Samples were then fixed in 100% acetone and cleared in 80% ethanol. All pictures were taken under a microscope (Olympus Instruments) equipped with a normal light source.

2.2.4.5 Confocal microscopy for subcellular localization of proteins

Imaging of tobacco leaf tissue was performed 48 h post-transformation (2.2.1.11.), using an inverted Zeiss laser scanning confocal microscope (LSM510 META; Zeiss, http://www.zeiss.com), and a 63× water immersion objective. For imaging expression of the GFP construct (pec100-GFP), YFP construct (ST-YFP) or both in leaf tissue, imaging settings were used as described in Brandizzi et al. (2002). The spectral properties of GFP (in pec100-GFP) allow efficient spectral separation from YFP (in ST-YFP) (Brandizzi et al., 2002a). Appropriate controls were used to exclude the possibility of energy transfer between fluorochromes.

2.2.5 *In silico* analysis and data processing

2.2.5.1 QTL analysis

The liquid culture grown RIL seedlings in presence of pectinases (endoPG/PME 0.5u/ml) were visually analyzed and the pictures were processed by ImageJ software (http://rsbweb.nih.gov/ij). Analyses of variance (ANOVA) were performed in Excel. The genetic information of the RILs was composed of 110 markers and provided by Prof. Thomas Altmann. Cosegregation of phenotypic properties and genetic markers was determined using WinQTL Cartographer version 2.5 (Basten et al., 1999; Zeng, 1993). The Kosambi function was used to transform recombination frequencies into centiMorgans. QTLs were identified using mean values obtained from ImageJ and a framework genetic linkage. Interval mapping was employed with an LOD score threshold of 3.0 to increase resolution and reduce background marker effect (Zeng, 1994). LOD thresholds were determined by computing 1,000 permutations for each trait (Churchill and Doerge, 1994).

2.2.5.2 Gene expression, co-expression and annotation analysis

Microarray data for gene expression was visualized by Genevestigator, a web based expression database and meta-analysis system (www.genevestigator.com Zimmermann P, Hirsch-Hoffmann M, Hennig L and W Gruissem, 2004). For co-expression analyses, ATTED-II (Arabidopsis thaliana trans-factor and cis-element prediction database) web based tool was applied (http://atted.jp/ Obayashi T, Hayashi S, Saeki M, Ohta H, Kinoshita K., 2009). Gene functional classification was analyzed by the Classification SuperViewer Tool

(http://bar.utoronto.ca). The normalized microarray data for the A. thaliana ecotypes were obtained from the Prof. Weigel's web site (www.weigelworld.org) and analyzed in Excel.

2.2.5.3 Protein sequence analysis

Nucleic acid and amino acid sequences were retrieved by searching public databases with the BLAST algorithm at TAIR (www.arabidopsis.org) and NCBI (www.ncbi.nlm.nih.gov). Subcellular localization and membrane topology was predicted using TargetP V1.1 (www.cbs.dtu.dk/services/TargetP), TMHMM V.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and the Aramemnon database (http://aramemnon.botanik.uni-koeln.de). The deduced amino acid sequences were aligned using the CLUSTALX program V.2 (Larkin MA et al 2007) with the default parameter settings. The phylogenetic tree was obtained with the neighbour-joining method with bootstrap values generated from 1,000 bootstrap samples and visualized by using the TreeView application (Page, R. D. M. 1996).

3 Results

3.1 Establishment of a pectinase based liquid culture screen of *A. thaliana*

The study of pectin biosynthesis and its function in plant development and defense against pathogens is still in its early stages. One way to overcome this gap is to identify plants with altered pectin structure and investigate the physiological changes that those structural changes bring with them. Here, a forward genetic screen was employed to identify such pectin mutants. The screen was developed by using fungal pectinases, endopolygalacturonase II (endoPG, EC 3.2.1.15), pectin methylesterase (PME, EC 3.1.1.11) and pectate lyase (PL, EC 4.2.2.2) from *Aspergillus sp.*. Those enzymes can be additives to a liquid plant culture to access Arabidopsis mutants with altered enzyme sensitivity, potentially due to alterations in pectin structure.

Before establishing a mutant screen based on the action of specific pectinases, the effect of this enzymatic influence on *A. thaliana* Col-0 had to be tested. For this reason, wild type seeds were placed, germinated and the resulting seedlings grown for two weeks in a liquid medium containing increasing concentrations of endoPG/PME or PL (fig.3.1, see 2.1.7). It was demonstrated that both treatments, endoPG/PME (fig.3.1. B, C and D) and PL (Fig3.1. E, F and G) lead to similar effects: a reduction in growth and root length, and at higher concentrations - a visible “chlorotic” syndrome within the two weeks of incubation.

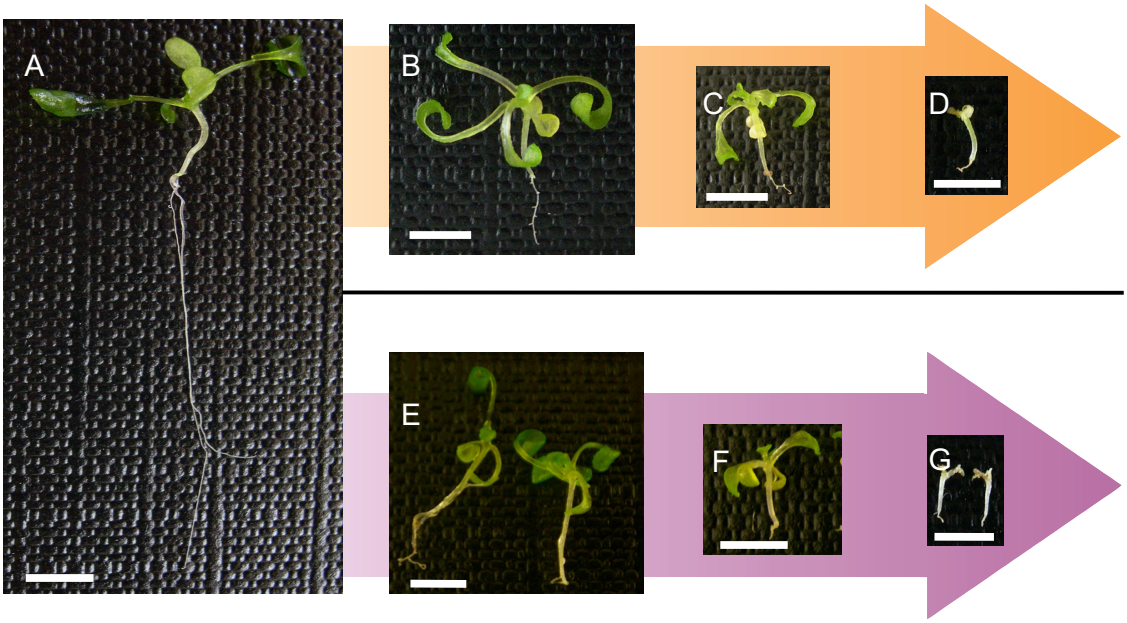

Figure3.1 Phenotype of *A. thaliana* seedlings incubated with pectinases in liquid culture. The effect of increasing concentrations of endoPG/PME (B. 0.1U/ml, C. 0.25U/ml and D. 2.5U/ml) and PL (E. 0.1U/ml, F. 0.25U/ml and G. 2.5U/ml) compared to a control treatment without any pectinase (A). Arrows indicate increase in enzyme concentration, orange – endoPG/PME, purple - PL. Scale bar = 0.5cm.

3.1.1 Effect on plant morphology

The effect that the pectinase incubation has on plant morphology during growth and development was investigated in greater detail. When endoPG/PME is applied at higher concentrations (2.5U/ml), it affects cell adhesion, represented by detachment of the plant tissue and its release in the liquid medium (fig. 3.2). In both hypocotyls and cotyledons there is a clear cell separation effect (C and D) compared to the control (A and D). The altered cell adhesion of these plants made them fragile and hard to handle. The process of cell separation occurring at high concentrations of pectinases is probably due to hydrolysis of the middle lamellae (the part of the cell wall that is between two adherent cells), which consists almost exclusively of pectinase hydrolysable pectic polysaccharides.

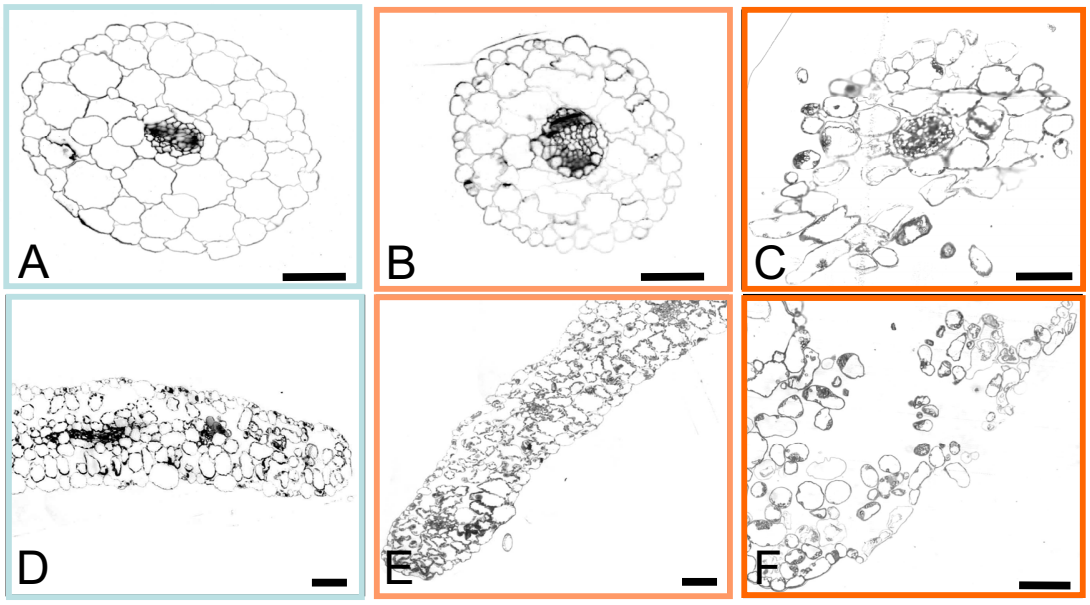

Figure 3.2. Light micrographs of cross-sections of plant tissues from liquid culture grown *A.thaliana*. The effect of increasing concentrations of endoPG/PME (B and E = 0.25U/ml C and F = 2.5U/ml) on hypocotyl anatomy (A, B, C) and cotyledon anatomy (D, E, F). Scale bar = 50µm (Data provided by Edouard Leboeuf).

3.1.2 Effect on cell wall structure

To further investigate the effect of this treatment on the structure of cell wall polymers, cell wall material derived from whole seedlings grown for two weeks in liquid culture with increasing concentrations of endoPG/PME was analyzed for monosaccharide composition (see 2.2.3.6.). From the acquired data it is evident that as expected the level of uronic acids is decreased with an increase of enzyme concentration (fig.3.3.). A potential compensatory effect by the plant cell was observed by the increase of the neutral sugars arabinose and galactose, which are the main constituents of rhamnogalacturonan I (RG-I) side chains – arabinan and galactan. Judging from the unchanged levels of mannose, fucose and xylose, there probably were no changes in the structure of the cross linking glycans.

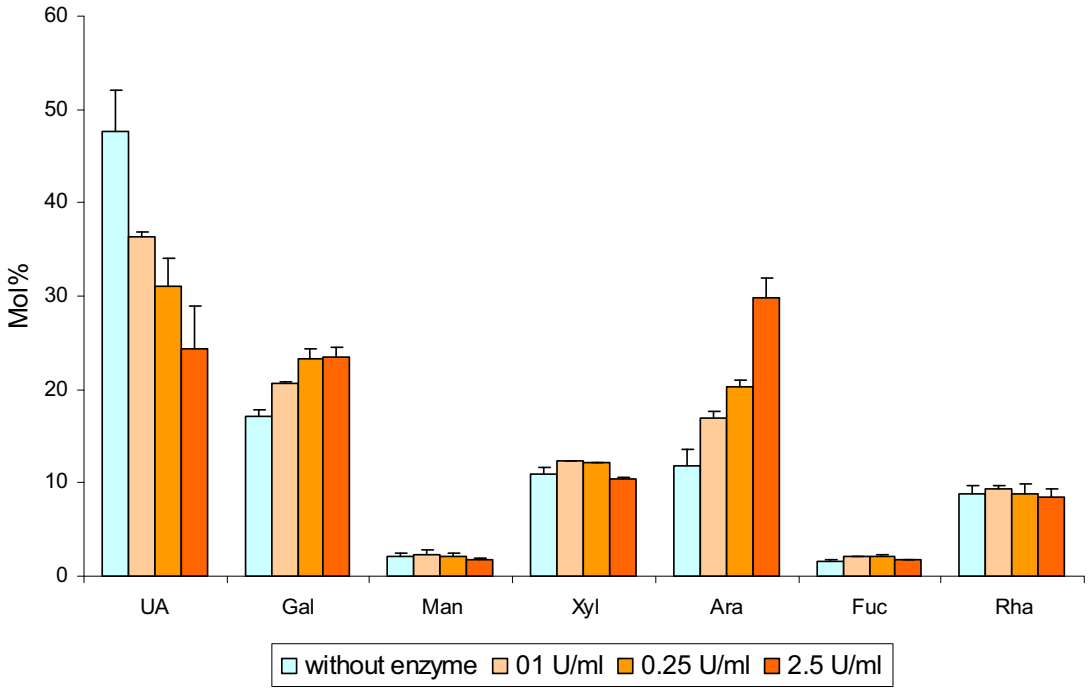

Figure 3.3. The effect of increasing concentrations of endoPG/PME (0.1U/ml, 0.25U/ml and 2.5U/ml) on the level of monosaccharides of alcohol insoluble residues. UA – uronic acids, Gal – galactose, Man – mannose, Xyl – xylose, Ara – arabinose, Fuc – fucose, Rha – rhamnose. Error bars indicate standard deviations, n=4.

The increase of cell wall arabinose and galactose with increasing concentrations of pectinases was further evaluated by purifying RG-I from cell wall material by an endoPG/PME digest (2.2.3.5.). Size exclusion chromatography of pectic polysaccharides solubilized from wall material resulted in a high molecular weight fraction containing RG-I again with an increased

abundance of arabinose and galactose, and expected decrease of uronic acids (fig.3.4.). This result confirms that the alteration in RG-I structure might compensate for the loss of homogalacturonan (HG) released by the pectinase-treatment.

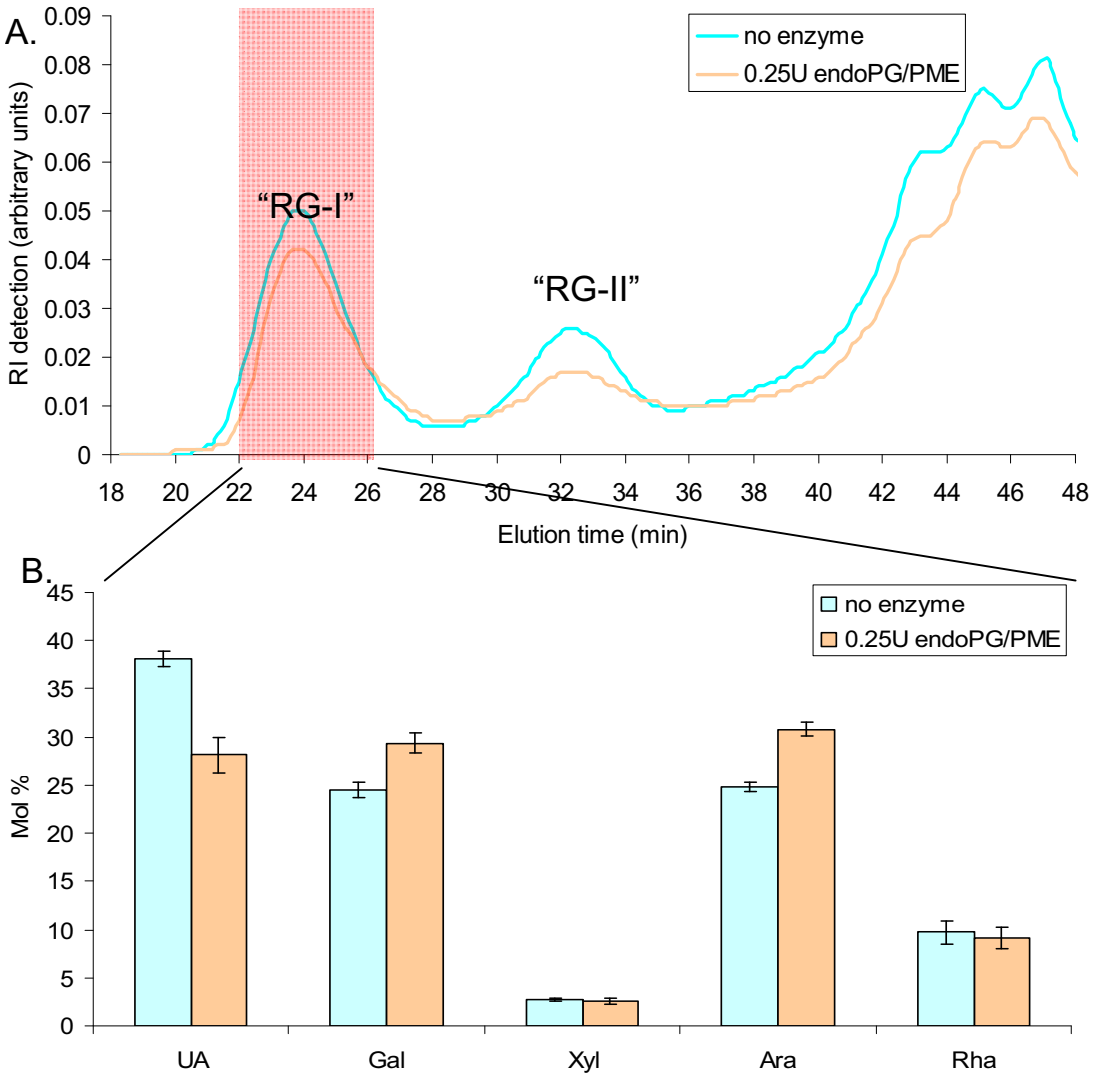

Figure 3.4. Analysis of RG-I from liquid cultured WT plants grown without and with pectinases (0.25U/ml endoPG/PME) A. Size-Exclusion Chromatography (SEC) using Refractive Index (RI) detection, of pectin fractions obtained from endoPG/PME digested cell wall material from liquid cultured plants grown without and with pectinases (0.25U/ml endoPG/PME). B. Monosaccharide composition analysis of the isolated RG-I fraction (elution time 22-26min, indicated as transparent red on A.) UA – uronic acids, Gal – galactose, Man – mannose, Xyl – xylose, Ara – arabinose, Fuc – fucose, Rha – rhamnose. Error bars indicate standard deviations, n=3.

To further investigate the cell wall changes, FTIR spectra were acquired from cell wall material of plants grown with 0.25 U/ml endoPG/PME and plants grown without enzyme (as described in 2.2.3.2.). Figure 3.5 shows the two averaged spectra (n=6) and their subtraction of cell wall preparations from whole seedlings of enzyme treated plant samples (0.25U/ml endoPG/PME) and control samples (grown without pectinase). Visual inspection of this subtracted spectrum reveals a decrease in the methyl-esterified HG (band at 1740 cm-1) as well as a decrease in the un-esterified HG (band at 1600 cm-1). This indicates that the enzymatic treatment affects not only the methyl esterified regions of HG, but also the un-esterified regions, leading to a total decrease in the abundance of the HG polymer, confirming the loss of uronic acids in figure 3.3. Also worth mentioning is the increase of wavenumber representing a C-O stretch in the polymers composed of neutral sugars, which might represent the observed increase in the monosaccharides arabinose and galactose (fig. 3.5.), but FTIR does not provide any specific information about the nature of the sugar.

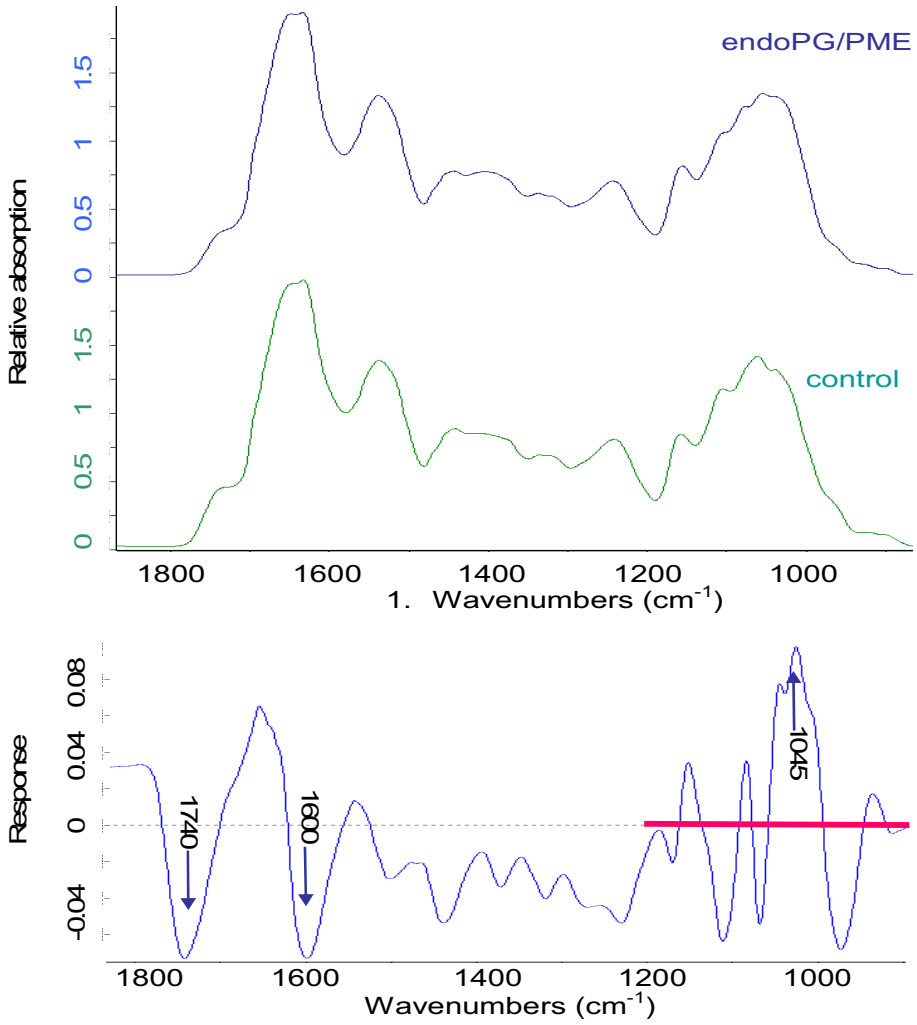

Figure 3.5 FTIR absorbance spectra of alcohol insoluble residues from plants grown with 0.25U/ml endoPG/PME and the control plants grown without enzyme. The carbohydrate fingerprint region (1200-900cm^{-1}) is indicated as a red baseline, arrows indicate the signature bands for ester groups of HG (1740 cm^{-1}), the salt state of unesterified HG (1600 cm^{-1}) and the C-O stretch of neutral sugars polymers (1045 cm^{-1}).

The degree of methyl-esterification of the pectic polysaccharides was determined by measuring the amount of released methanol from the alcohol insoluble material upon treatment with alkali (as described in 2.2.3.8. and 2.2.3.9.). These measurements are presented in figure 3.6, showing a decrease of total methyl-esters in the endoPG/PME treated samples (0.25 U/ml) which thus led to decrease in the degree of methyl-esterification.

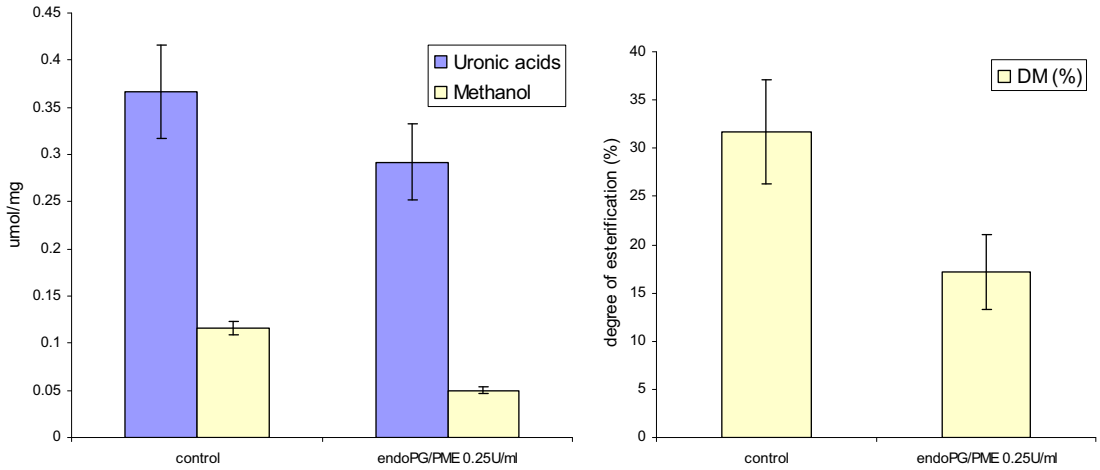

Fig.3.6 Total levels of uronic acids and methanol and degree of methyl-esterificaton of the cell wall material isolated from *A. thaliana* Col-0 grown on liquid culture without and with 0.25U/ml of endoPG/PME. DM indicated degree of methyl-esterification (mol %). Error bars indicate standard deviation, n=3.

We also used immunohistochemical methods to investigate the specificity and the pattern of pectin methylesterification in plant tissues (as described in 2.2.4.2.). Transverse hypocotyl sections from control and treated plants were stained with a monoclonal antibody (JIM7) recognizing heavily methyl esterified regions of HG (Willats, W. G. et al. 2000). Upon plant growth in the endoPG/PME solution (0.25 U/ml), this epitope is only present in the stele and disappears in the cortex and the epidermis. This suggests that the endoPG/PME treatment leads to de-esterification of HG in the radial tissues as detected by the antibody that are easily exposed to the surrounding media, and not the stele, where the enzyme apparently is not able to penetrate. This may be due to the Casparian strip which stops the passive apoplastic flow of components such as water and solutes into the stele of the plant (fig.3.7).

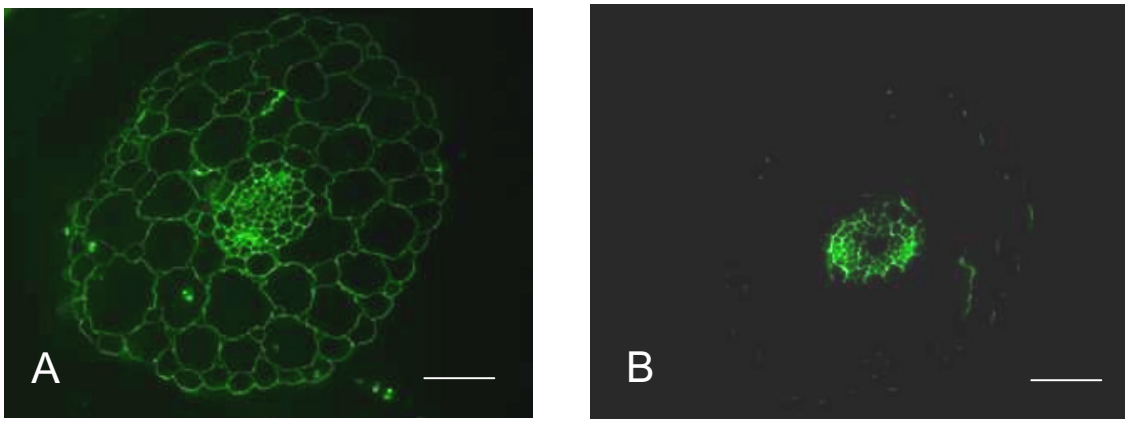

Figure 3.7 Antibody labeling (JIM7) of methyl-esterified HG of transverse sections of hypocotyls from liquid culture grown *A. thaliana*. A. *A. thaliana* Col-0 grown on liquid media without any enzyme. B. *A. thaliana* Col-0 grown with endoPG/PME (0.25 U/ml each). Scale bar = 25 µm.

In conclusion, growth of seedlings in an enzyme solution leads to a visible change in plant morphology and structural changes of pectic polysaccharides, allowing the assessment of this assay for a forward genetic screen, where plant mutants with altered polysaccharide composition could be identified on basis of their response to the enzyme treatment. We speculate that if the presence of pectinases affects the growth and development of the plants in liquid culture and this correlates to changes in pectin structure, then mutants with altered pectin structure would display also alterations in the visible phenotype upon this treatment. In order to explore this possibility, the pectinase based liquid culturing was further assessed on previously characterized *A. thaliana* cell wall mutants as well as mutants affected in disease resistance.

3.1.3 The effect of pectinases on liquid culture grown characterized mutants of *A. thaliana*

As a "proof of concept" for mutant screening conditions, the liquid culture influence of pectinases on known cell wall related mutants was tested. Here the identified powdery mildew resistant mutants (*pmr*), the *murus* mutants (*mur*) and *rhm2* mutant were tested.

The *pmr* (powdery mildew resistant) mutant lines were isolated in a forward genetic screen of plants that do not support normal growth of the powdery mildew pathogen, *Erysiphe cichoracearum*. The mutants thus represent a collection of plants affecting genes required for a compatible interaction between a plant and a pathogen (Vogel, J. and Somerville, S. 2000).

These mutant lines are used as a tool to assess if the established liquid culture screen could provide mutants that are affected in the pathogen induced defense reactions.

As seen in Fig.3.8.B, the mutants *pmr2* and *pmr5* are reduced in size, showing that they could have been isolated by the pectinase based screen. *Pmr5* also features a reduction in root growth when compared to plants grown without pectinases (Fig.3.8. A). Only *pmr6* did not show any visual alterations, even with both treatments (endoPG/PME and PL). Perhaps because of its resistance to powdery mildew, this mutant also shows no susceptibility to the endoPG/PME or PL treatment. These data indicate that some of the previously identified mutants, involved in plant - pathogen interaction can be also identified by the pectinase based liquid culture screen.

The *murus* mutants were isolated on a basis of structural difference in the monosaccharide composition of the cell wall material (Reiter, W. D. et al. 1997). These mutants can be used to test the screening approach for finding structural cell wall mutants. 11 different loci, designated *mur1 to mur11*, were identified by this approach. In this trial we observed that the most susceptible mutant was *mur11* (Fig.3.8. B), a mutant that has not been genetically characterized, but shows complex changes in monosaccharide composition: reduced amounts of rhamnose, fucose and xylose in cell wall material (Reiter, W. D. et al. 1997). Slight morphological differences were observed in *mur1, mur3* and *mur6* grown with endoPG/PME in liquid culture, represented by reduction in growth for *mur1* and *mu6*, and chlorotic leaves for *mur3* (Fig.3.8. B). *mur3* also had the most abnormal phenotype when incubated with PL; it showed a reduced size and browning of the seedling (Fig.3.8. C). The observed phenotype in *mur3/kam1* could be due to its function in the secretory pathway maintanance (Tamura, K. et al. 2005). *Mur4* and *mur5* did not show visible alterations upon treatment with endoPG/PME or PL. MUR4 besides being involved in arabinosylation of glycans has a sugar signaling role, and therefore may not be involved in pathogen related signaling. The genetic locus of *mur5* is still not determined, and there is also no data on the resistance of the mutant to pathogens.

A knock-out in RHM2, a gene that encodes a UDP-L-rhamnose synthase, was also tested. This enzyme is converts UDP-glucose to UDP rhamnose, which is required for the synthesis of the RG-I backbone (Usadel, B. et al. 2004). This line also showed a slight reduction in growth when subjected to the pectinase containing liquid culture.

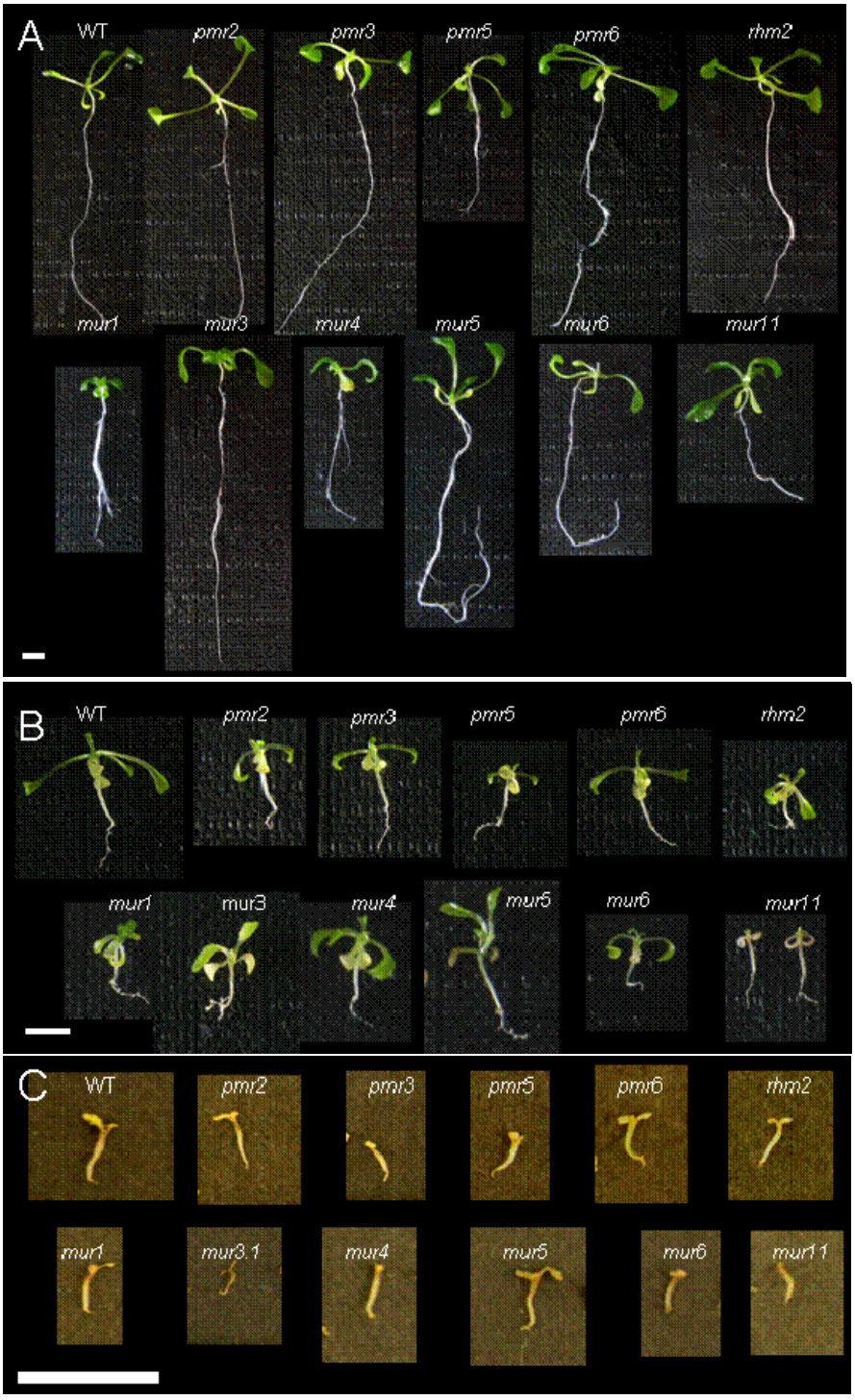

Figure 3.8 Effect of pectinases on liquid culture grown characterized cell wall mutants of *A. thaliana*. Scale bar =1cm A. Plants grown in liquid culture without any enzyme, B. Plants grown in liquid culture in the presence of endoPG/PME (2.5U/ml), C. in presence of PL (40U/ml).

Taken together, these data indicate that the methodology (growth of seedlings in liquid culture containing 0.25U/ml endoPG/PME) allows the selection of mutants that are affected in genes required for plant-pathogen interaction and structural cell wall features, in particular pectic polysaccharides.

3.2 Identification of novel pectin related mutants through a pectinase based liquid culture screen

The methodology, based on the established liquid culture system with pectinases was employed to screen chemically and insertional mutagenized seed pools as well as natural variations of *A. thaliana* for the identification of conditional pectin mutants that might not have been identified otherwise, as their change in pectin structure might have been subtle, restricted to certain tissue types, lethal, and/or conditional, i.e. the phenotype is only present when the plant is subjected to pectinase "stress". The screening strategy for pools of randomly mutagenized seed lines was conducted in two steps, consisting of a primary and a secondary screen (Fig. 3.9.). First, the pools were subjected to liquid culturing in presence of pectinases for two weeks, where the primary mutants could be selected on the basis of visual morphological alterations compared to the wild type (WT) *A. thaliana* plants. The aim of the primary screen was to rapidly pick up plant lines representing potential pectin mutants. The morphological phenotype was scored visually and the selected lines were rescued by continuing growth on soil for seed production. The next seed-generation was submitted to a secondary screen; some of the seeds were grown again in liquid culture containing pectinases to confirm the visual alteration observed in the primary screen. Some of the seeds were grown in culture without any enzyme addition to verify that the observed phenotype is due to the addition of the enzyme and not a developmental phenotype *per se*.

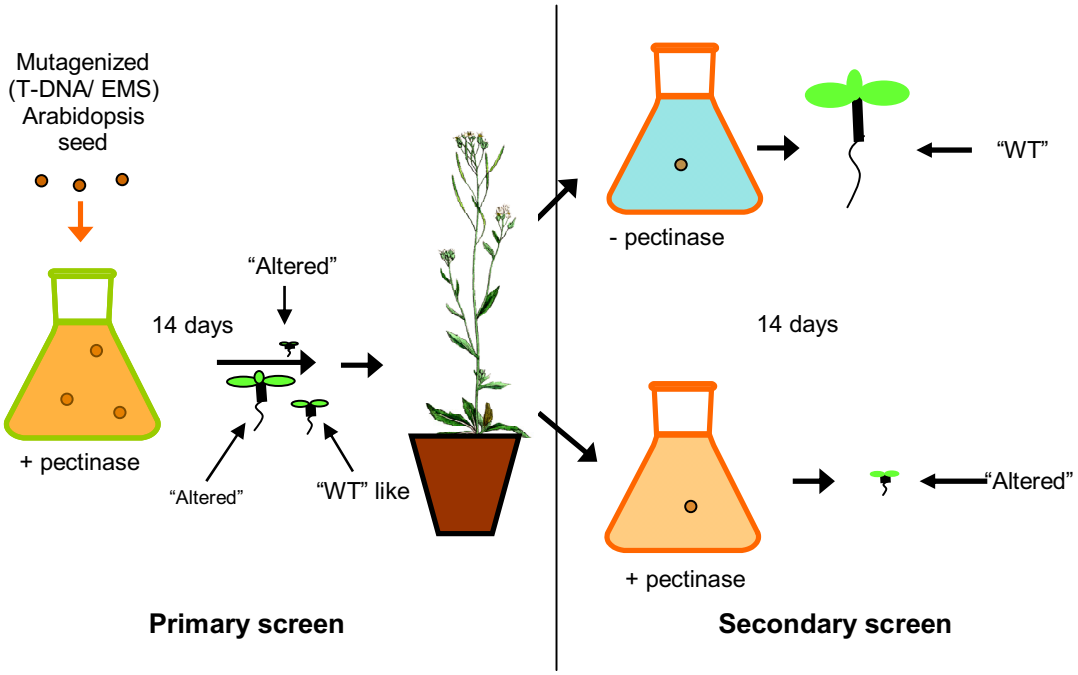

Figure 3.9 Screening strategy for the selection of potential pectin mutants using pectinases in liquid culture. Mutagenized *A. thaliana* seeds are germinating and grown for 14 days in the presence of pectin degrading enzymes and selected on the basis of their morphological alterations (Primary screen). The selected lines are propagated and the next seed-generation is submitted to the same conditions (+ pectinase) and in addition to control conditions without the enzyme (- pectinase), verifying that the morphological alterations are specific due to the presence of the pectin degrading enzymes.

3.2.1 Genetic screen of *A. thaliana* T-DNA tagged populations

A. thaliana seed pools mutated by an activation tagging approach were used for the screen. It is expected that those mutants contain loss or gain-of-function mutations, depending of the insertion site of the T-DNA. The activation tagged lines were based on the transformation of Col-0 plants with the T-DNA vector pSKI15 containing BASTA resistance for selection purposes (see 2.1.6 and (Weigel, D. et al. 2000).

Pools of lines containing approximately 300-350 independent T-DNA insertion lines were subjected to the screen per flask. In the primary screen, a total of approximately 10.000 individual transformants were screened for altered visual phenotype as described in 3.2. Approximately 800 plants (~8% of all transformants) were selected and transferred to soil set for seed amplification. Of those seedlings app. 10% of the plants (79 plants) did not survive the transition to soil, and app.1% of the survived selected plants (12 plants) did not produce seeds, but seeds were obtained from the remaining 708 lines. All of these lines were subjected to the

secondary screen, but only in 35 cases could the phenotype be unambiguously confirmed. After the secondary screen, these 35 lines were identified and genotyped (Appendix Table 8.1, overview of the screened pools and the number of confirmed insertional mutants).

3.2.1.1 Identification of the T-DNA insertion site

In order to determine the site of the T-DNA insertion, thermal asymmetric interlaced (TAIL)-PCR was utilized. This method is a hemispecific PCR amplification protocol that combines nested, insertion-specific primers with arbitrary degenerate primers, to amplify the genomic DNA flanking the T-DNA left border. The left border of the T-DNA was chosen as bait because literature evidence indicates the loss or inconsistency in the right border of the T-DNA during the insertion event. We applied the recently developed high throughput mTAIL-PCR method, developed by Syngenta (Sessions, A. et al. 2002) and described in 2.2.1.6.. This modified TAIL-PCR allows faster amplification of the flanking genomic region. Typically, six reactions with a T-DNA border primer and each of six arbitrary degenerate (AD) primers are used per round of PCR to maximize the likelihood of generating a product. To increase the efficiency of TAIL-PCR, a pool of four arbitrary degenerate primers were used per reaction, and a two round PCR was performed instead of three (Appendix; Fig. 8.1.). Figure 3.10 shows typical T-DNA left border secondary TAIL-PCR products produced with the pool of four AD primers. This reaction usually produces several PCR products, but these products were easily distinguished in the subsequent sequencing. The TAIL-PCR products were sequenced with a distal T-DNA left border primer and the sequencing results were aligned to The Arabidopsis Information Resource (TAIR) version 7 of the Arabidopsis genome by Basic Local Alignment Search Tool (BLAST).

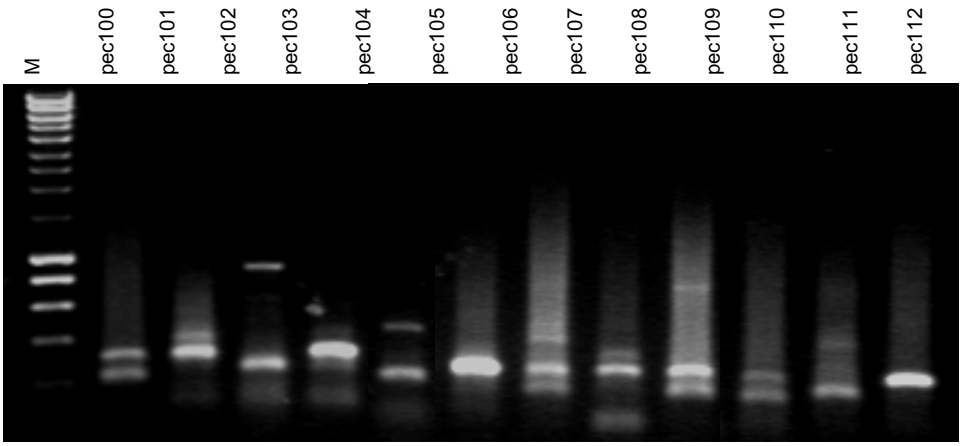

Figure 3.10. Secondary TAIL-PCR products from examples of T-DNA insertion lines. M - DNA ladder (100bp), pec100-pec111 – TAIL PCR results from different T-DNA insertion lines

To confirm the assigned putative insertion site gene specific primers were used in combination with the T-DNA left border specific primer. This approach was also used for obtaining homozygous insertion line in the cases where the parental line was heterozygous (see 2.1.2.2). The TAIL PCR results could not be confirmed for three lines (*pec109*, *pec119* and *pec125*), probably because of an artifact TAIL PCR product, and for one line (*pec109*) no homozygous progeny could be obtained. Because of these reasons, these lines were not used in further study. Two lines had insertions in intergenic regions (*pec102* between At3g17630 and At3g17640, *pec133* between At1g15970 and AT1G15980); therefore we assumed they would not disrupt the gene function. For 7 T-DNA lines, a second homozygous allele could not be identified (*pec101, pec103, pec105, pec116, pec118*, *pec126* and *pec127*), further analysis on this lines was also abolished. The other confirmed lines (22 out of 35) with their assigned number (pec#) and the genes that are affected by the insertion are presented in table 3.2. A detailed table with the insertion site and alleles and all data obtained from this lines is presented in the appendix (see Appendix, table. 8.2 and fig. 8.2).

In order to prioritize the list of genes for further studies, all the genes identified in this screen were submitted to bioinformatic predictions for protein subcellular localizations using the SubCellular Proteomic Database (SUBA, http://www.plantenergy.uwa.edu.au/suba2, (Heazlewood, J. L. et al. 2007) (see Table 3.1.). From this prediction genes were prioritized based on the following: first priority were gene products predicted to be localized to the Golgi apparatus, the sight of pectin biosynthesis, of second – gene product predicted to be

extracellular, where they could influence pectin modification and turnover. Of third priority were genes localized to the plasma membrane – putative protein kinases that could be involved in pectin mediated signaling.

Table 3.1 List of identified T-DNA lines. The genes with subcellular prediction relevant to the cell wall are color coded: blue – prediction of localization in the Golgi apparatus, yellow – on the plasma membrane, orange – extracellular.

pec #	Gene ID	Gene functional annotation	Predicted subcellular localization
pec100	At2g44280	lactose permease, putative sugar transporter	Golgi apparatus
pec104	At2g40000	unknown protein having Hs1pro-1 domain	Mitochondria
pec106	At1g76740	cell wall-anchored protein, subtilisin-like serine protease	Extracellular
pec107	At4g31680	transcriptional factor B3 family protein	Nucleus
pec108	At2g31800	ankyrin protein kinase	Mitochondria
pec110	At5g09360	LAC14, laccase, multicopper oxidase	Extracellular
pec111	At3g14225	GLIP4, carboxylic ester hydrolase	Plastid
pec112	At5g40910	disease resistance protein (TIR-NBS-LRR class)	Plasma membrane
pec113	At1g24320	Alpha-glucosidase, putative; similar to GCS1 GH63	Cytosol
pec114	At1g58370	RXF12 xylanase	Extracellular
pec115	At1g11545	xyloglucan endotransglucosylase/hydrolase protein 8	Extracellular
pec117	At1g05240	peroxidase, putative	Extracellular
pec120	At5g01030	Transcriptional factor tubby, unknown protein	Nucleus
pec121	At1g09950	transcription factor-related; similar to ZW2	Nucleus
pec122	At4g35830	aconitate hydratase, citrate hydro-lyase / aconitase	Cytosol
pec123	At5g40470	LRR protein, similar to F-box family protein	Extracellular
pec124	At1g50110	branched-chain amino acid aminotransferase	Cytosol
pec128	At5g14890	NHL repeat-containing protein, Soluble quinoprotein glucose dehydrogenase	Plastid
pec129	At1g26240	proline-rich extensin-like family protein	Extracellular
pec130	At3g59830	ankyrin protein kinase	Plastid
pec131	At5g18990	PME, pectinesterase family protein	Extracellular
pec132	At2g39440	unknown protein PIG-P	Extracellular

To further investigate the functional role of the above 24 genes identified by the screen, Classification SuperViewer was applied. The list of genes was analyzed using the MAtDB database (MIPS Arabidopsis thaliana Database, www.mips.gsf.de/proj/plant/jsf/athal, (Schoof, H. et al. 2004). A ranking score was calculated for each functional class, which may be better than absolute numbers of genes in a given cluster falling into each functional classification

category, since this score shows the abundance of the functional classes (Provart, N. and Zhu, T. 2003). The input set is bootstrapped 100 times to provide some idea of over- or under-representation reliability. From this analysis it can be concluded that the majority of the identified genes are in a functional category related to the cell wall (Fig.3.11.). Such data augment that the described screening approach yielded a high proportion of cell wall mutants.

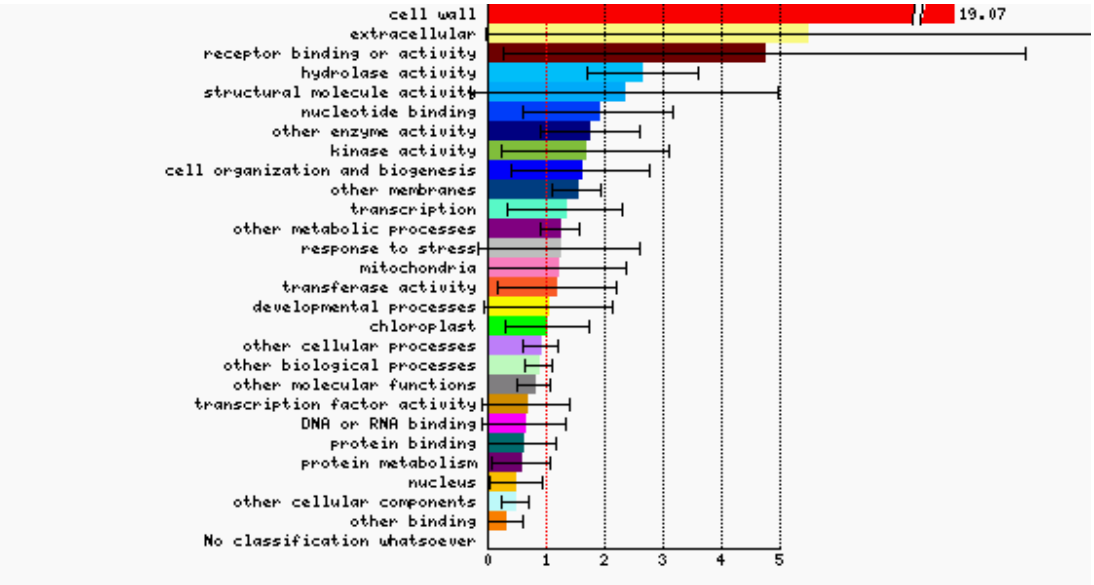

Figure 3.11 Classification of 24 *A. thaliana* genes identified by the genetic screen using "Classification SuperViewer" Normalized to frequency in *A. thaliana* set (± bootstrap standard deviation) from BAR (http://bar.utoronto.ca).

At a closer look, the genes that were assigned as cell wall related can be subdivided in different categories. There is one gene; *PEC112* that encodes a leucine rich repeat protein kinase that may be involved in perception of external signals, such as oligogalacturonides (OGA) signaling or sensing the cell wall integrity. *PEC117* is a putative apoplastic peroxidase involved in oxidative coupling of extracellular polymers. We have also identified two apoplastic proteases; *PEC105* encoding an aspartyl protease family protein and *PEC106* – a subtilase (subtilisin-like serine protease), presumably active in the cell wall. In this list there is also a pectin modifying enzyme: *PEC131*, a pectin methyl-esterase which possibly modifies the methyl esterification of HG. There are also two cell wall degrading enzymes: *PEC114* (xylanase, hydrolyzing O-glycosyl compounds) and *PEC115* (xyloglucan:xyloglucosyl transferase). These genes, together with the genes putatively localized in subcellular

compartments related to pectin biosynthesis and modification (the Golgi apparatus, the plasma membrane and the cell wall) are a good resource to study the nature of their involvement in pectin biogenesis, metabolism and signaling. The rest of the genes range from transcription factors to metabolic enzymes; these genes are not likely to be directly involved in pectin biosynthesis, but are still interesting candidates that may regulate or modulate pectin metabolism. We focused our attention on one of these genes, *PEC100,* a putative sugar transporter. The identification and characterization of the corresponding mutant is presented in the next chapter.

3.2.2 Identification and characterization of *pec100* – a putative sugar transporter involved in pectin metabolism

Pec100 is one of the identified T-DNA lines from the pectinase based liquid culture screen. It was identified because of a severe morphological phenotype; 14d old plants exhibit underdeveloped and bleached cotyledons when compared to the WT (Fig.3.12). When the mutant is grown in liquid culture without endoPG/PME it displays a WT-like phenotype, thus fulfilling our criteria for a potential pectin mutant. Genetic mapping by TAIL-PCR revealed a T-DNA insertion in the 5'UTR of *At2g44280* (Fig.3.15. A). This gene encodes a putative sugar transporter. Its closest homolog in *A. thaliana* is a protein, similar to a lactose permease-related protein (*At3G60070*). PEC100, the putative sugar transporter has a length of 462 amino acids, a calculated molecular weight of 50.9 kDa, and an isoelectric point of 7.9. It belongs to a large gene superfamily of transporters called Major Facilitator Superfamily (MFS) general substrate transporter superfamily, encompassing 161 members. This sugar transporter is predicted to contain 11 transmembrane domains by ARAMEMNON (Schwacke, R. et al. 2003) and is predicted to be localized in the secretory pathway (PrediSi, (Hiller, K. et al. 2004) and www.ccb.imb.uq.edu.au/Golgi, (Yuan, Z. and Teasdale, R. D. 2002). *Pec100* is therefore an interesting candidate for further studies, because it might influence pectin structure ore biosynthesis.

3.2.2.1 Morphological study of *pec100*

To prove that the T-DNA tag in PEC100 was responsible for the morphological phenotype in liquid culture, a second allele was identified and analyzed. This second allele was a T-DNA tagged line obtained from the SALK collection (SALK_139158) designated as *pec100-2*. When grown in liquid culture in the presence of endoPG/PME the phenotype was the same as *pec100-1* (Fig3.12.).

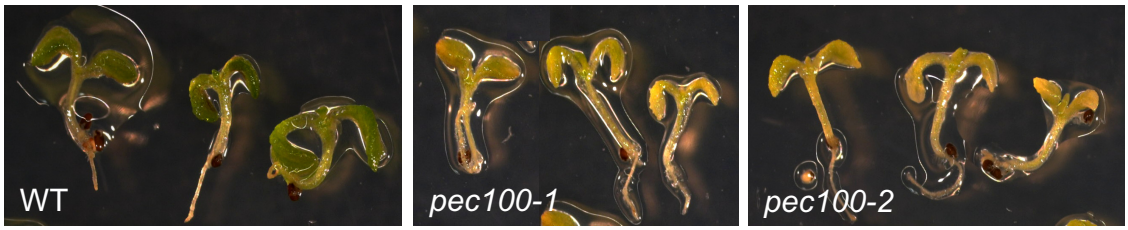

Figure 3.12. *A. thaliana* seedings grown in presence of 2.5U/ml endoPG/PME.

Both of these lines showed no alteration in development when grown without enzyme in liquid culture or on soil, in normal conditions, indicating that the phenotype observed in liquid culture is due to the presence of the enzymes (Figure 3.13.). This indicates that *pec100-1* and *pec100-2* are conditional mutants.

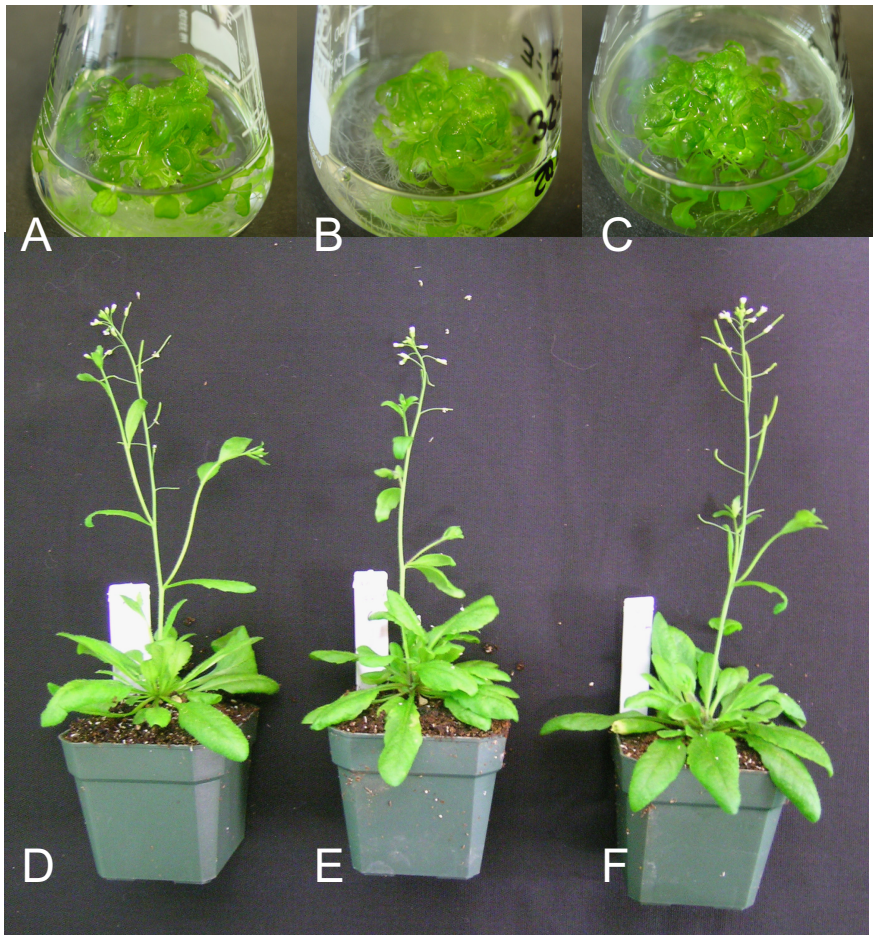

Figure 3.13. Photographs showing WT, *pec100-1* and *pec100-2* plants grown in liquid culture without the enzymes and on soil under controlled conditions. The *pec100* alleles do not show any abnormal morphology and development when compared to WT. A. Liquid culture of WT, B. *pec100-1*, C. *pec100-2*. D. 5-week-old WT grown on soil, E. *pec100-1*, F. *pec100-2.*

3.2.2.2 Insertion sight and transcript analysis of *pec100*

The precise T-DNA insertion sites of the two *pec100* alleles was determined by amplifying the flanking genomic region and sequencing using a left border T-DNA specific primer (see 2.1.2.1). These data show that the T-DNA insertion in *pec100-1* is located in the 5'UTR region after nucleotide 164 after the start codon, and for *pec100-2* is inserted in the first intron after nucleotide 684 after the start codon (Fig. 3.14.).

RT-PCR analysis was utilized to estimate the transcription level of *At2g44280* in these lines (see 2.2.1.9.). No transcript was detected in *pec100-2*, and a strong decrease of the transcript was observed for *pec100-1* (Fig.3.14. B). This decrease may be due to the fact that the T-DNA insertion is in the 5'UTR region of the gene.

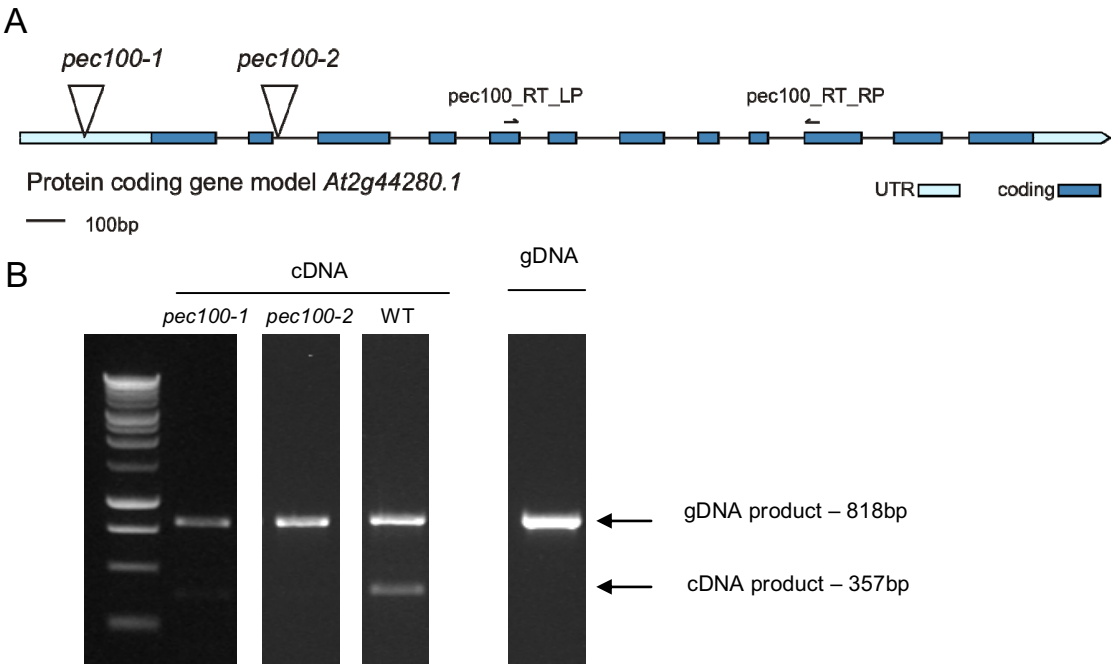

Figure 3.14. Characterization of *pec100* alleles. A. Genomic map indicating the T-DNA insertion sites (*pec100-1* and *pec100-2*) and the primers used in RT-PCR on the gene model At2g44280.1. B. Gel electrophoresis showing the knock down and knock out of At2g44280 transcript in *pec100* alleles.

3.2.2.3 Cell wall analysis of *pec100*

Cell wall material from both *pec100* alleles (obtained from one week old whole seedlings grown under 16 h light regimes) was analyzed for their neutral and acidic monosaccharide composition (see 2.2.3.7.). This analysis indicated changes in RG-I structure, a decrease in rhamnose and arabinose, representing backbone and sidechain, respectively (Fig.3.15.).

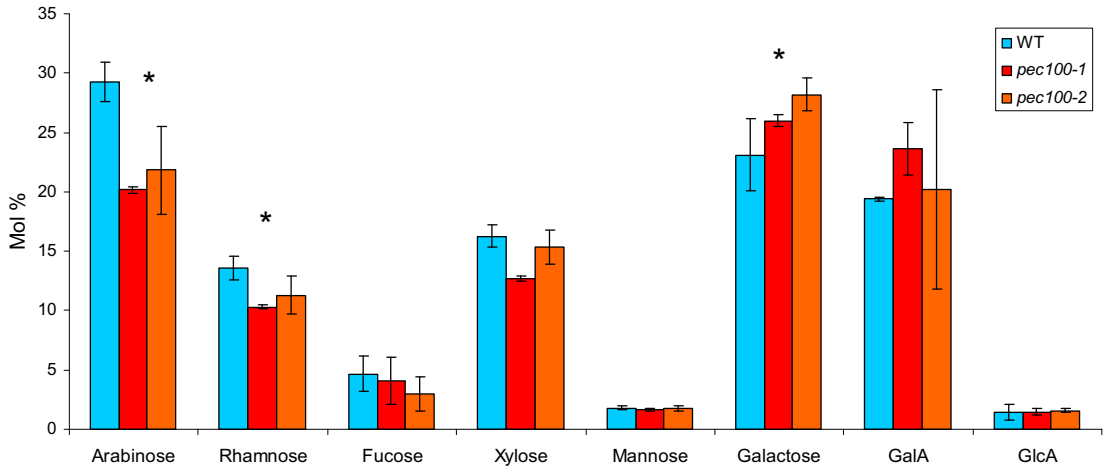

Figure 3.15. Monosaccharide composition of cell wall material from *pec100* lines and WT. Data presented as mol percent, GalA – galacturonic acid, GlcA – glucuronic acid, error bars represent standard deviation, (n=3).

3.2.2.4 Protein localization and promoter activity of *PEC100*

In order to gain more insights into the function of the *PEC100* gene, topology prediction, protein localization and promoter activity were studied. The putative transporter encoded by *PEC100* has 12 transmembrane domains as predicted by the ARAMEMNON database (www.aramemnon.botanik.uni-koeln.de), which utilizes 17 different topology prediction algorithms (Fig.3.16.).

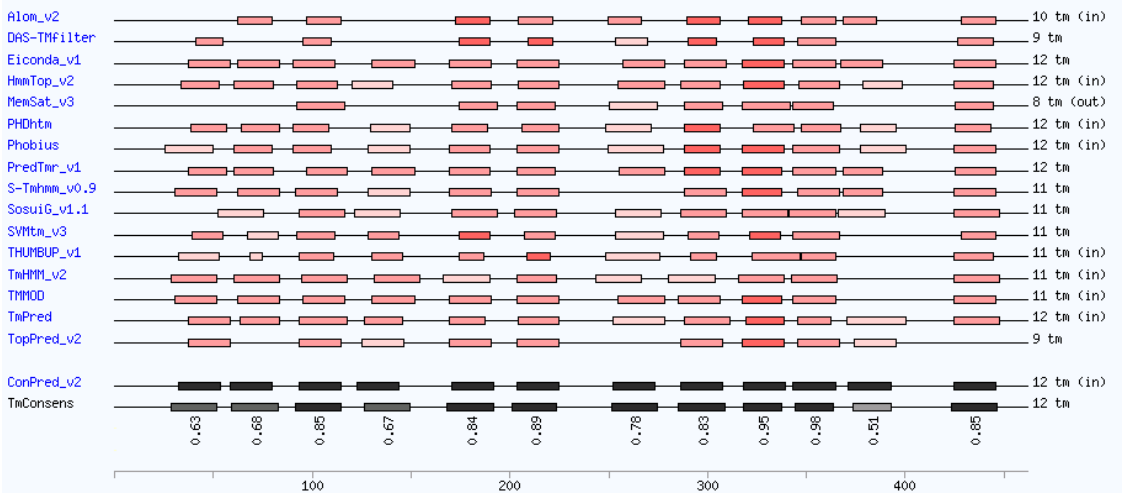

Figure 3.16 Consensus prediction of the TM topology for *PEC100* (*At2g44280*) by ARAMEMNON. The prediction analysis identified 12 probable TM domains. Numbers below the predicted TM domains indicate their hydrophobicity scores.

In order to study its subcellular protein localization, a GFP fusion construct was generated. In brief, the At2g44280 full length cDNA clone was obtained from TAIR (pENTR221-At2g44280) and cloned into pMDC83 (a N-terminal GUS fusion vector; 2.1.3). This vector was co-expressed transiently with a Golgi marker (ST-YFP) obtained from Prof. Brandizzi (for details on the ST-YFP construct see 2.1.3. and Brandizzi et al., 2002) in tobacco epidermal cells. Confocal microscopy analysis (as described in 2.2.4.5) shoved a clear co-localization with the Golgi marker, indicating a localization of PEC100 protein in the Golgi apparatus (Fig. 3.17.)

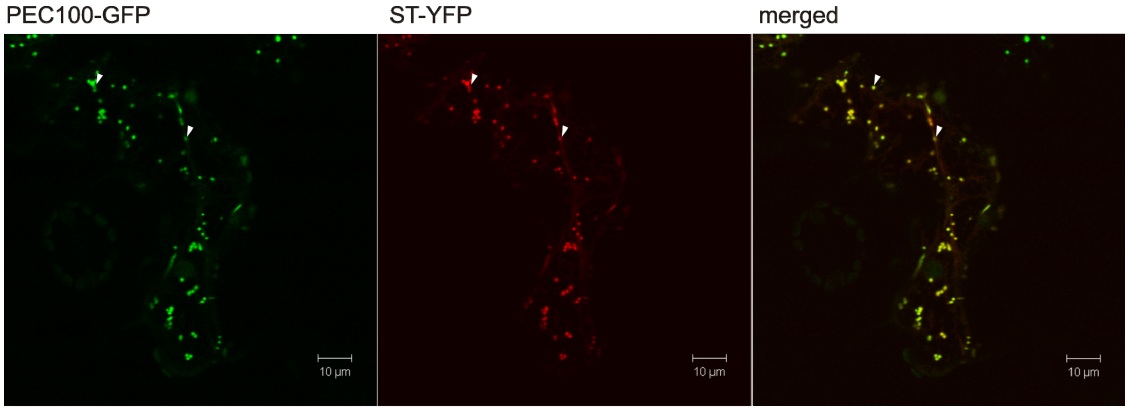

Figure 3.17 Co-localization of *PEC100* with the marker for the Golgi apparatus (ST-YFP) in tobacco epidermal cells. A. Construct expressing the full length cDNA of At2g44280 fused to GFP at the C terminus. B. ST-YFP marker for the Golgi apparatus. C. Overlay of the two channels. Arrows indicate two examples of co-localization to the Golgi apparatus.

The level of *pec100* transcript could not be evaluated by existing gene expression data since its ORF is not represented on any A. thaliana microarray chip, limiting possible data mining efforts and *in silico* expression analysis. Therefore, the expression pattern of this gene was analyzed by a *promoter::gusA* analysis. A vector construct containing a fusion between the promoter fragment of At2g44280 (ranging from the end of the previous gene, At2g44270 to the start codon of At2g44280) and *GUS* reporter gene in pMDC164 (see 2.1.3). This construct was used to produce transgenic *A. thaliana* plants. The obtained transformants were analyzed for their GUS marker expression (as described in 2.2.4.4.). In etiolated seedlings the expression of *pec100* promoter was restricted to the elongation zone at 4 days of growth, which suggest a presence of the gene-product during cell elongation (Fig.3.18. A). In addition, strong GUS

activity was observed when seedlings were grown under light conditions; the promoter activity was much stronger and present ubiquitously in all plant tissues (Fig.3.18. B). At this early stage of development cell division is the predominant process, and cell elongation is not strongly active. One explanation is that expression of this gene is light regulated.

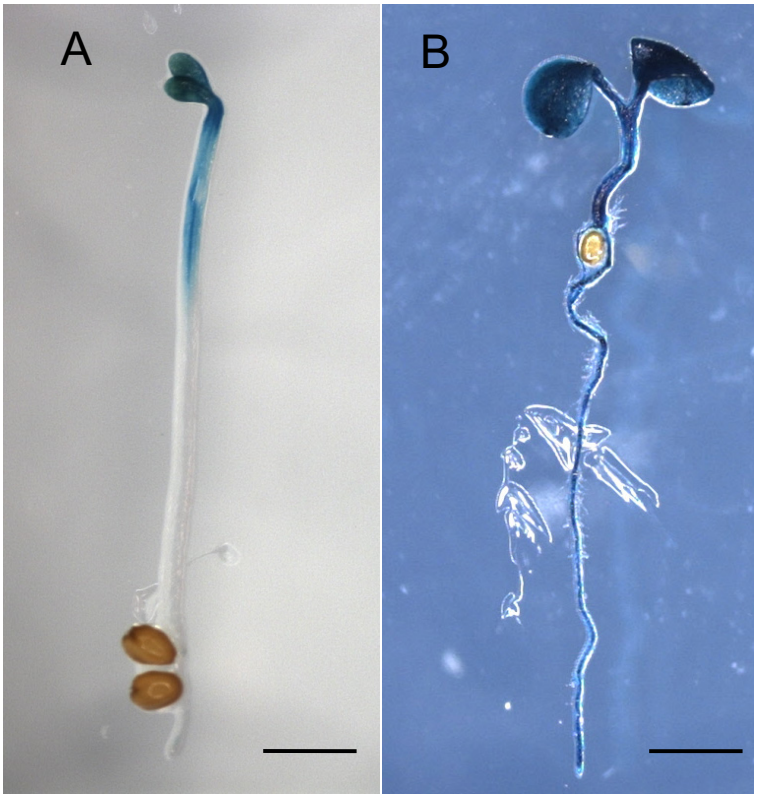

Figure 3.18 Expression of *pec100 promoter::gusA*. A. 4 day old etiolated seedling, B. 7-day old light frown seedling. Scale bar = 1mm.

In summary, *pec100-1* was a T-DNA line identified by the pectinase based liquid culture screen. It affects a gene that encodes a putative sugar transporter, presumably active in the secretory pathway. The observed phenotype was confirmed with a second allele, *pec100-2.* Both alleles showed a change in cell wall composition related to RG-I (decrease of cell wall bound arabinose and rhamnose). *PEC100* is highly expressed ubiquitously in light grown seedlings and in the elongation zone of the etiolated seedlings. Protein localization studies showed accumulation of the protein in the Golgi apparatus, suggesting that it might play a role in shutteling pectin related sugars into/ out of the Golgi apparatus.

3.2.3 Genetic screen of *A. thaliana* EMS mutagenized populations

To broaden the spectrum of mutants, the screening methodology involving endoPG/PME was extended to an EMS mutagenized *A. thaliana* seed-population. EMS mutations may lead to a complete (if a codon is mutated to a stop codon) or partial loss of gene function (an amino acid change) or, less frequently, to a change of function (Greene, E. A. et al. 2003).

In this work an M2 generation of an EMS-mutagenized Arabidopsis seed population was used. Approximately on average 8 mutations were present per line (personal correspondence with Prof. Thomas Altmann). On the basis of this mutation rate, the M2 sample population necessary for identifying a mutation per 1 kbp gene with probability of 99% should be a size of at least 10.000 seeds. We applied the "seed mixture" method, consisting of seed pools from bulked M2 progenies (Redei, G. P. 1975). Approximately 50 seeds from a M2 pool were tested together, which represented 80% of the mutations. Five pools were bulked in a superpool (50 seeds/pool, total of 250 seeds) and grown in one flask. Initially 355 out of 391 pools, corresponding to 18.000 seeds, were screened and 369 mutants were selected in the primary screen and placed on soil. Out of these 369 mutants 275 survived and gave progeny (94 T-DNA lines did not survive when transplanted to soil or were infertile). The secondary screen confirmed 24 potential mutants, which had an altered phenotype when submitted to pectinases and have no changes when grown without enzyme in liquid culture. These alterations include morphological indicators of stress: "bleached" cotyledons, browning of the seedlings, and reduced growth and organ development. Some examples of mutant phenotypes are presented in Fig.3.19. Here, *pec5* and *pec30* have highly browned cotyledons, and *pec31* has "bleached" leaves. Photographs of all the other lines and their cell wall monosaccharide composition are presented in the appendix (see Figs 8.3).

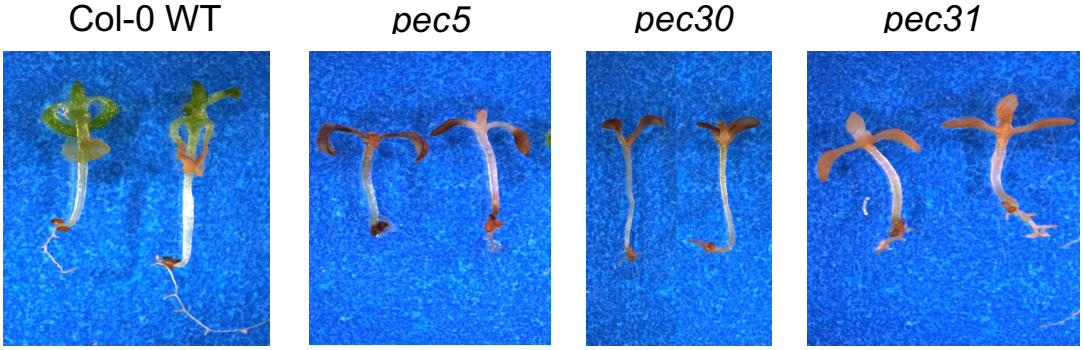

Figure 3.19 Examples of mutant phenotypes observed when grown in liquid culture with endoPG/PME

In the next stage of selection the 24 selected mutants were tested for alterations in their cell wall chemistry. The cell wall material from 4 day old etiolated seedlings was analyzed for its monosaccharide composition by GC-MS of alditol acetate derivatives (see 2.2.3.6). These data are presented in the appendix, fig. 8.3. Out of the 24 mutants analyzed, 7 didn't show any alterations in the neutral monosaccharide composition of the cell wall. These 7 mutants were designated as putative"signaling" mutants, since they were succeptible to the pectinase incubation, but no detectable structural changes were detected on global cell wall analysis. The other 17 mutants displayed statistically sidnificant deviations in the cell wall monosaccharide composition from the WT. These mutants represent the putative structural class, having broad spectrum of changes - from altered abundance of only one monosaccaharide to complex changes of several monosaccharides in the same time (see fig. 8.3 for getails). One such mutant (*pec5*) is described further in more detailed manner.

3.2.3.1 Analysis of *pec5* - a putative pectin mutant

Based on FTIR analysis *pec5* exhibits a decrease in arabinogalactan and methyl-esterified HG related spectral bands; however the spectral bands for un-esterified HG were not increased (band at 1414-1420 cm^{-1}) (Fig.3.20).

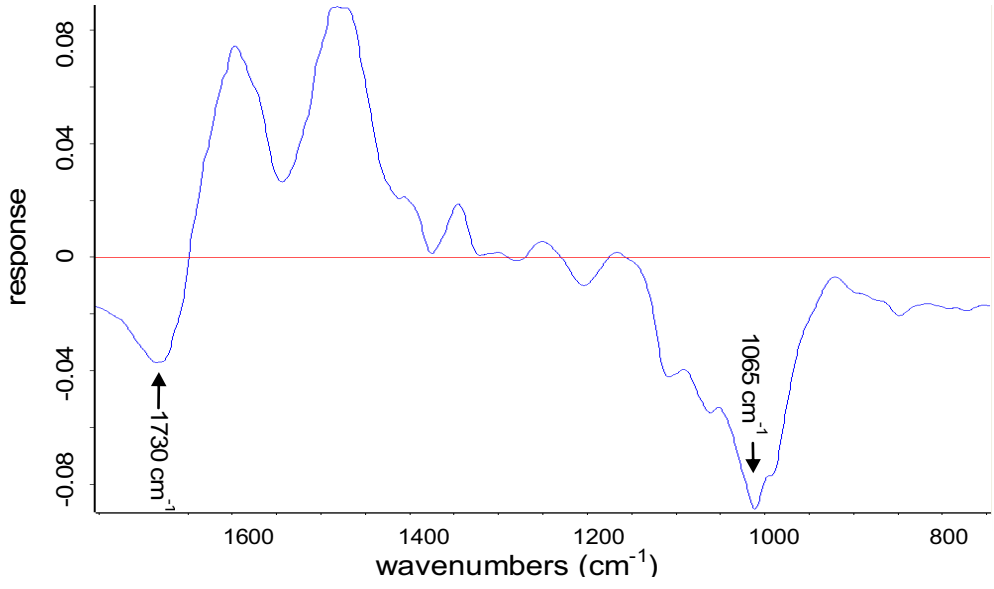

Figure 3.20 Subtracted FTIR spectrum of *pec5* from the WT spectrum obtained from cell wall material of dark grown seedlings. The two negative peaks represent a decease in the carboxylic esters of HG (Martin, J. A. et al. 2005), 1730 cm^{-1}) and in arabinogalactan (Robert, P. et al. 2005), 1065 cm^{-1}). Average of n=3.

The analysis of the monosaccharide composition of wall materials obtained from *pec5* and WT also indicated a decreased arabinose content in *pec5* (Fig.3.21). This data, together with the FTIR analysis suggested that *pec5* might have an altered RG-I abundance and/or composition.

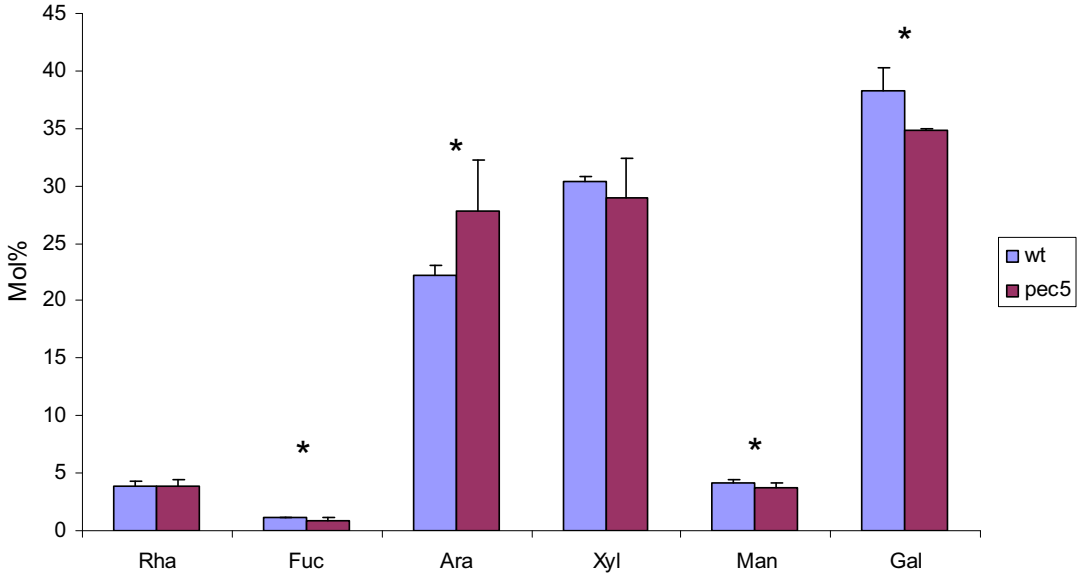

Figure 3.21 Monosaccharide composition of cell wall material from WT and *pec5* plants. Data presented as molar percentage, error bar represents standard deviation, n=6, asterisks indicate statistically significant differences ($p<0.05$).

The fractions corresponding to RG-I polymers were analyzed for their monosaccharide composition in order to estimate the RG-I structure, and confirm that the RG-I structure is altered in *pec5* (Fig.3.22). RG-I was extracted as described in 2.2.3.5 and as depicted on fig. 3.4. This data shows that the RG-I polymer of *pec5* has a compositional difference; an increase in arabinose and rhamnose and a decrease in galactose (Fig.3.22).

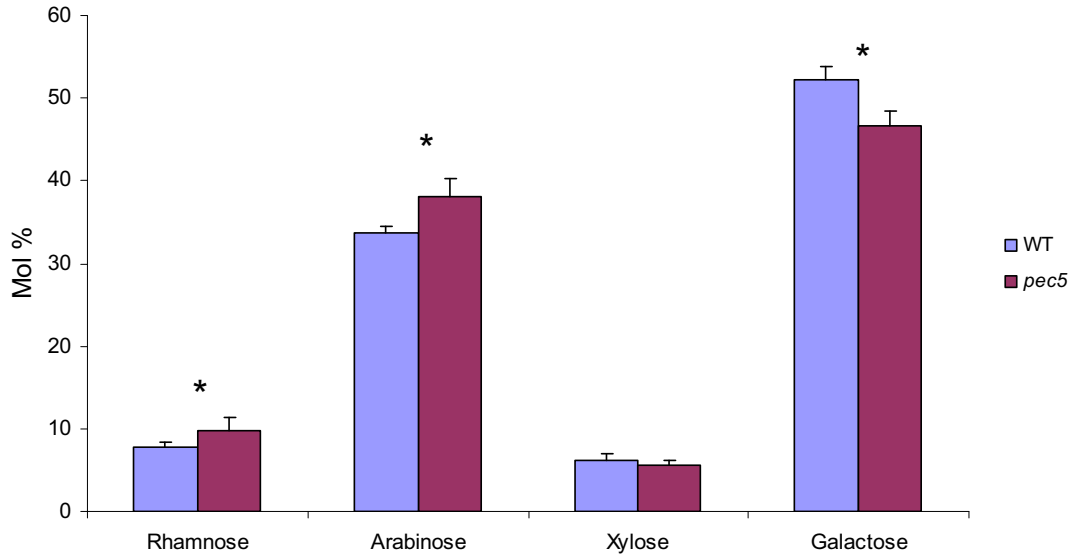

Figure 3.22. Monosaccharide composition of RG I fractions (Mol%) isolated by size exclusion chromatography of endoPG/PME treated cell wall material from 4 day old etiolated seedlings. Data presented as molar percentage, error bar represents standard deviation, n=4, asterisks indicate statistically significant differences ($p<0.05$).

3.2.3.2. Polymorphic marker analysis of *pec5*

In an attempt to map the responsible mutation in *pec5*, a segregating F2 population was produced of a cross of *pec5* with Landsberg erecta. However, this analysis clearly demonstrated that *pec5* is in a C24 background (Fig, 3,23).

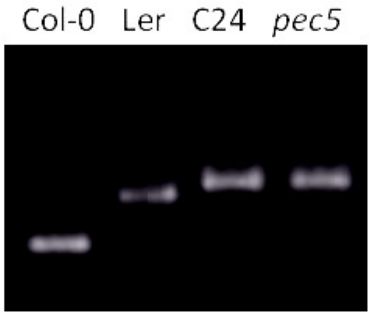

Figure 3.23 SSLP marker analysis of WT Col-0, Ler, and WT C24 compared to pec5. Marker NGA111 was utilized, which produces PCR amplicons with different sizes (Col-0 – 128bp, Ler – 162bp, C24 - 180bp).

In summary, the reverse genetic screen using EMS mutagenized lines resulted in identification of several potential pectin mutants, one of which was partly characterized. *Pec5*

displays a striking morphological phenotype when grown in liquid culture containing pectinases, and was selected on the basis of cell wall alteration. It has an altered RG-I composition; a decrease in the content of RG-I side-chain galactosyl residues. Positional cloning attempts of the mutation indicated that *pec5* is in a C24 background. C24 could partially be responsible for the pec5 phenotype, and hence a potential natural variation in the pectin structure of Col-0 and C24 was investigated.

3.3 Liquid culture screen of natural variations in Arabidopsis

3.3.1 Phenotypical differences between Col-0 and C24

C24 displays a severe response to the pectinases: it is strongly chlorotic when grown in liquid culture in the presence of pectinases and has a strong growth arrest at the stage of fully opened cotyledons, when Col-0 is at the stage of rosette leaves emergence (Fig.3.24.).

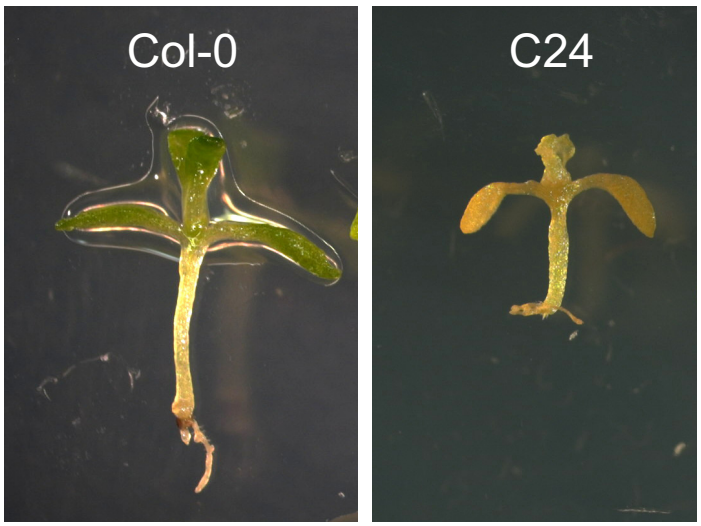

Figure 3.24 Liquid culture phenotype of *A. thaliana* ecotypes grown in presence of 2.5U/ml of endoPG/PME for 14 days.

Liquid cultured C24 in presence of pectinases also shows an ectopic accumulation of cell wall phenolic compounds at the tip of the cotyledons when observed under UV light (see 2.2.4.3.), perhaps as a defense response (Fig.3.25.).

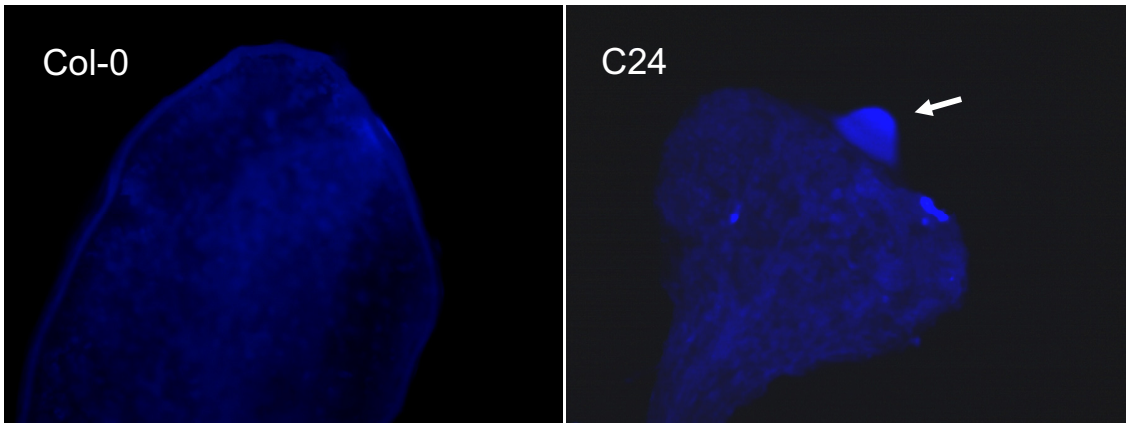

Figure 3.25 Fluorescent microscopy phenotype of intact cotyledons from two *A. thaliana* ecotypes grown in presence of 2.5U of PG/PME for 14 days. Arrow indicates accumulation of UV fluorescent phenolic compounds at the tip of the cotyledon in C24.

C24 exhibits morphological differences compared to Col-0 when grown on soil. It develops more stems per plant, and the rosette leaves have a different morphology: they are wavy and serrated; the rosette size is smaller (Fig.3.26).

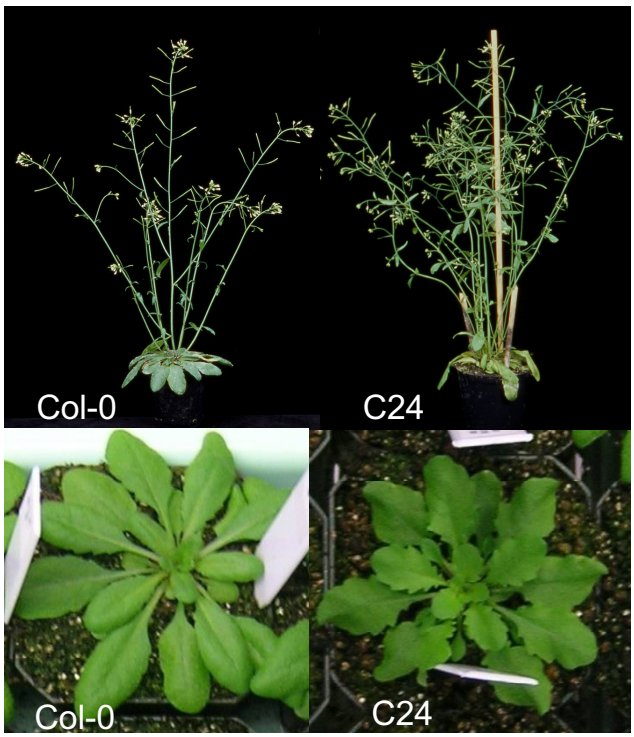

Figure 3.26 Morphological phenotypes of Col-0 and C24 after 6 weeks of growth (top) and after 2 weeks of growth (bottom).

3.3.2 Cell wall structural analysis of C24 and Col-0

In order to determine the differences in cell wall structure of C24 and Col-0, detailed analysis of the cell wall composition and especially pectin structure were conducted. The monosaccharide composition was determined by GC-MS analysis of TMS derivatives from etiolated seedlings of Col-0 and C24 ecotypes (as described in 2.2.3.7.). TMS derivatization was employed to quantify and distinguish the two uronic acids present in cell wall polymers: galacturonic and glucuronic acids. It can be observed that C24 has a significant decrease in the galacturonic acid content, indicating a difference in the abundance of HG (Fig.3.27).

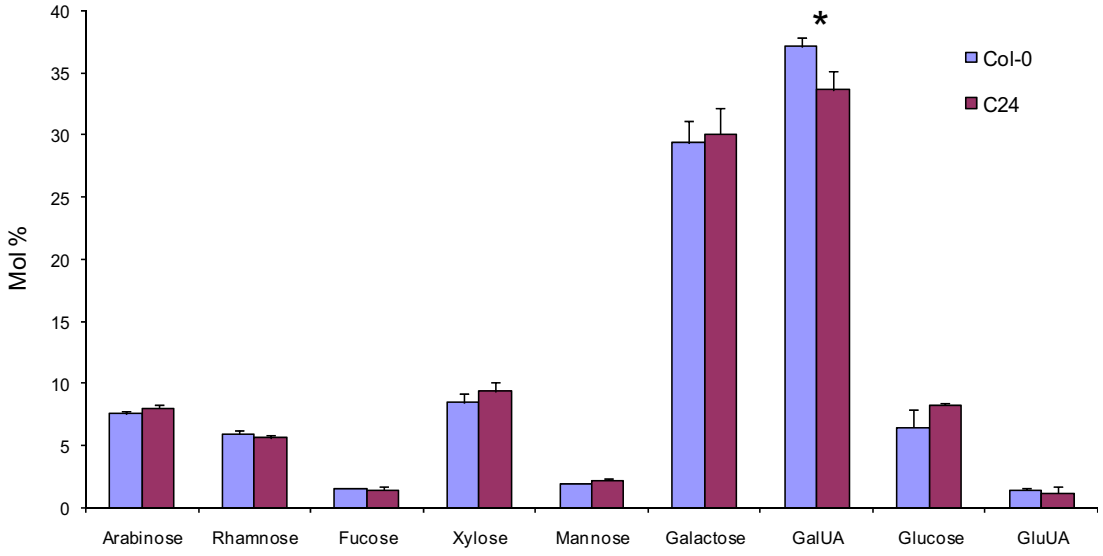

Figure 3.27 Monosaccharide composition of total cell wall material from etiolated seedlings of the two ecotypes. Data presented as a molar percent, error bars indicate standard deviation (n=6), asterisks indicate statistically significant differences ($p<0.05$).

Since the main difference in the total cell wall composition was the levels of galacturonic acid, a detailed analysis of the pectic polysaccarides was performed by oligosaccharide mass profiling (OLIMP) of oligogalacturonides (OGAs) released upon digestion of cell wall material with pectinases (endoPG/PME) (see 2.2.3.12.). This methodology helps elucidate the HG structure/accesability by measuring the relative abundance of OGAs with different structures. Upon digestion of HG, C24 produced less unesterified digalacturonides and

trigalacturonides, but more methyltetragalacturonides and acetyldimethylpentagalacturonides when compared to Col-0 (Fig. 3.28) OLIMP of pectic fragments released from cell wall material from Col-0 and C24 of dark grown seedlings indicated that the two ecotypes differ in the structure of HG or accessibility to the pectinases, possibly because of the difference of the pattern of HG methyl esterification.

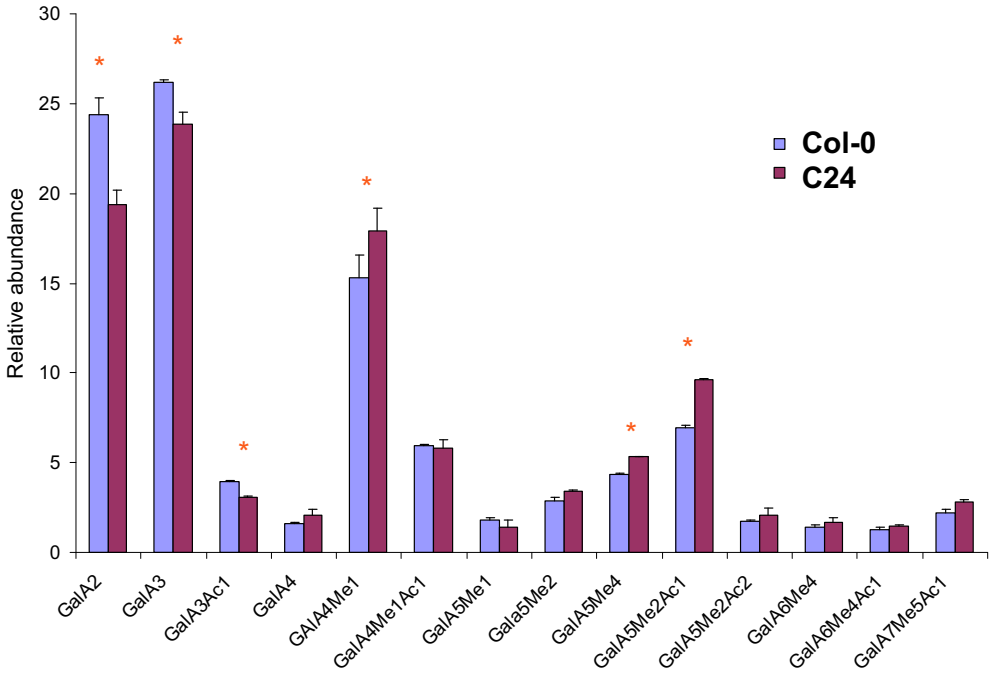

Figure 3.28 Oligogalacturonide mass profiling of ePG/PME digested cell wall material grown Col-0 and C24. GalA – galacturonic acid, Me – methyl ester, Ac – acetyl ester, numbers indicate GalA monomers and esters. Data presented as average ± standard deviation of n=6 samples. Red asterisks indicate significant difference ($p < 0.05$).

3.3.3 Immunohistochemistry of Col-0 and C24

The HG structural differences between Col-0 and C24 were also probed with monoclonal antibodies directed against different HG epitopes (LM7, JIM5 and JIM7) on hypocotyl transverse sections of 4-d-old etiolated seedlings (as described in 2.2.4.2.). These monoclonal antibodies were chosen because of their epitopes specificity towards different degrees of methyl esterification, LM7 binds to a non-blockwise pattern of HG de-esterification, JIM5 binds to low methyl-esterified HG and JIM7 to high methyl-esterified HG. Hence, used in

combination, these antibodies can give a view of the overall methyl-esterification status of HG on a tissue-level. There were differences in the labeling patterns and intensities between Col-0 and C24 (Figure 3.29). LM7, which recognizes partly un-esterified HG (tetrameric galacturonide flanked by methyl-galacturonic acid) labeled C24 in a different pattern compared to Col-0. This labeling pattern had stronger labeling in distinct cell wall areas (ectopic thickenings in the cortex), whereas in Col-0 this was not as evident and the labeling was more uniform. The epitope for JIM5 has not been precisely determined, although it is known that this antibody binds to low methyl-esterified HG. Using JIM5 the labeling was also different between the two ecotypes, not in pattern but in intensity (Fig. 3.29). C24 was less labeled in the cortex and epidermis, but had relatively similar labeling in the stele. The strongest deviation was observed with JIM7, an antibody that binds to high-esterified HG. Whereas C24 showed a strong ubiquitous labeling, Col-0 labeling was specific for the stele (Fig.3.29).

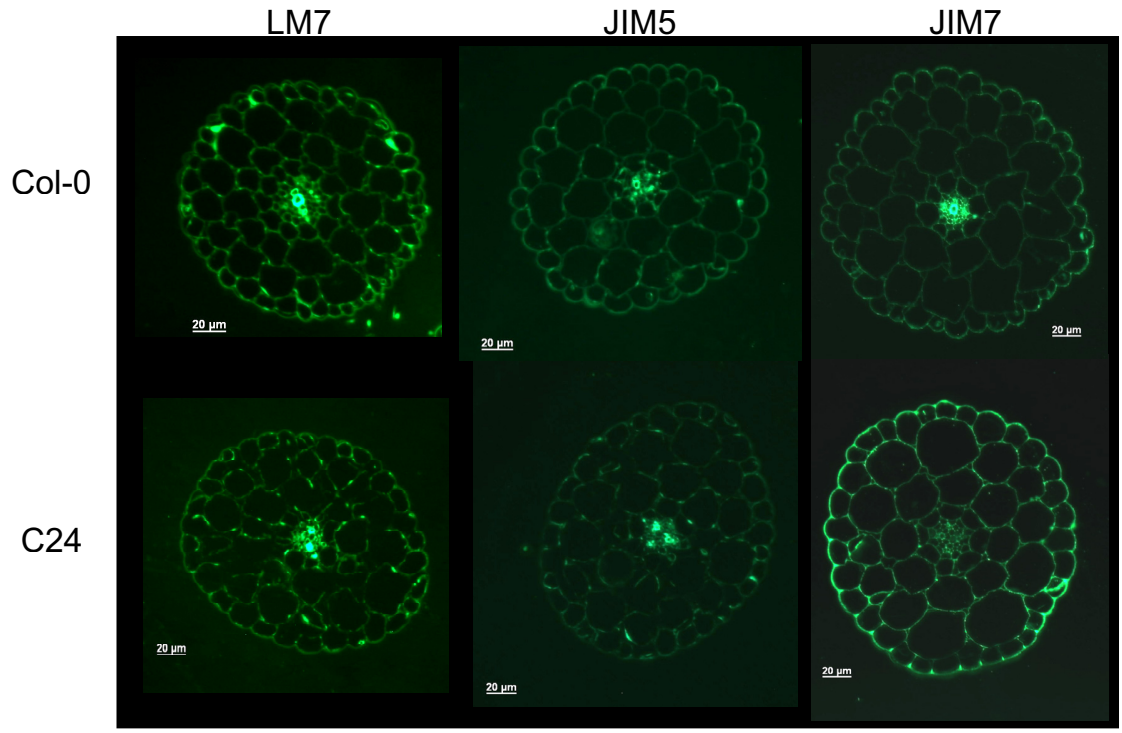

Figure 3.29 Immunofluorescent labeling of Col-0 and C24 with different HG-directed antibodies. Scale bar is 20 um

Taken together, the cell wall analytical results indicate that Col-0 and C24 have a different HG abundance and methyl-esterification status. A previous study showed that the degree of HG methyl-esterification correlates with the susceptibility of plants to some bacterial

pathogens (Lionetti, V. et al. 2007). Therefore, pathogen induced stress was studied in Col-0 and C24.

3.3.4 Pathogen induced stress reactions in Col-0 and C24

The induction of an oxidative burst is correlated with the response of plants to pathogen invasion, and also occurs when plant cell cultures are treated with elicitors - pathogen produced molecules that trigger a hypersensitive reaction (HR) within the plant (Bowles 1990; Hahlbrock et al. 1995; Lamb & Dixon 1997). Pectin degrading enzymes of fungal origin can release pectin derived elicitors and induce production of reactive oxygen species (ROS). In order to study the effect of this type of elicitation in the two ecotypes, ROS levels were measured after the addition of endoPG. Leaves from Col-0 and C24 were treated with endoPG as an elicitor and ROS accumulation was monitored (see 2.2.2.3. for details). Leaf pieces of *A. thaliana* ecotypes C24 and Col-0 responded with the rapid release of ROS when treated with endoPG, however the response of C24 was two-fold higher, compared to Col-0. This suggests an increased sensitivity to the fungal endoPG in C24 (Fig.3.30).

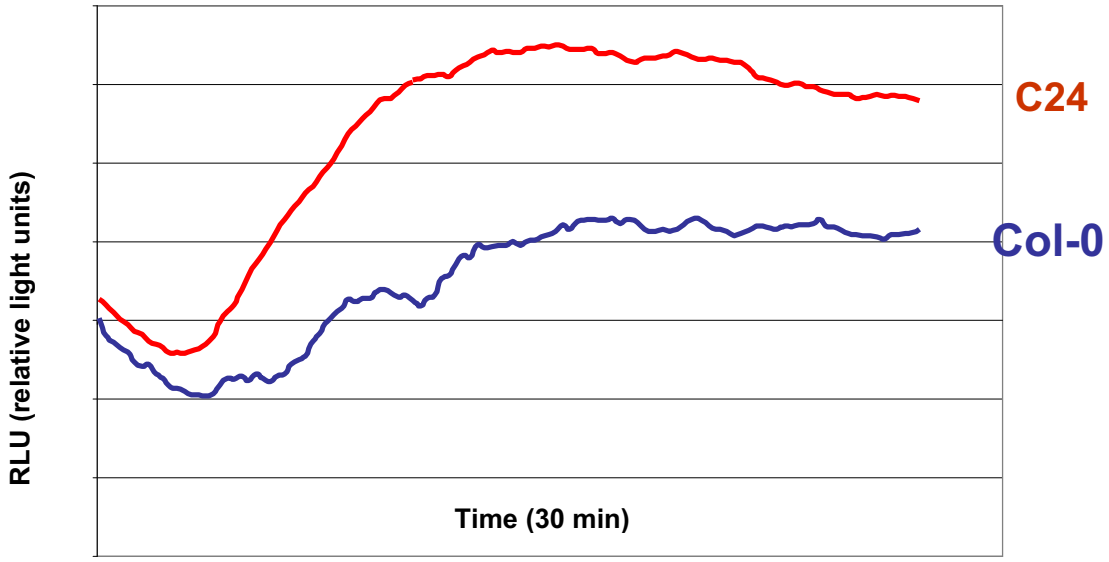

Figure 3.30 Oxidative burst in the leaf tissues of ecotypes Col-0 and C24. Luminescence of *A. thaliana* leaf slices in a solution with luminol and peroxidase after treatment with 10 µm/ml endoPG.

Pathogenicity tests were performed on leaves of both ecotypes with *Alternaria brassicicola*, a cabbage fungal pathogen (see 2.1.5). This pathogen causes the dark leaf spot disease on most *Brassica* species, and is also active on *A. thaliana,* under controlled conditions. The pathogenicity test produced different responses in the two ecotypes. In Col-0 it developed one necrotic lesion, while in C24 several small necrotic spots were observed (Fig. 3.31)

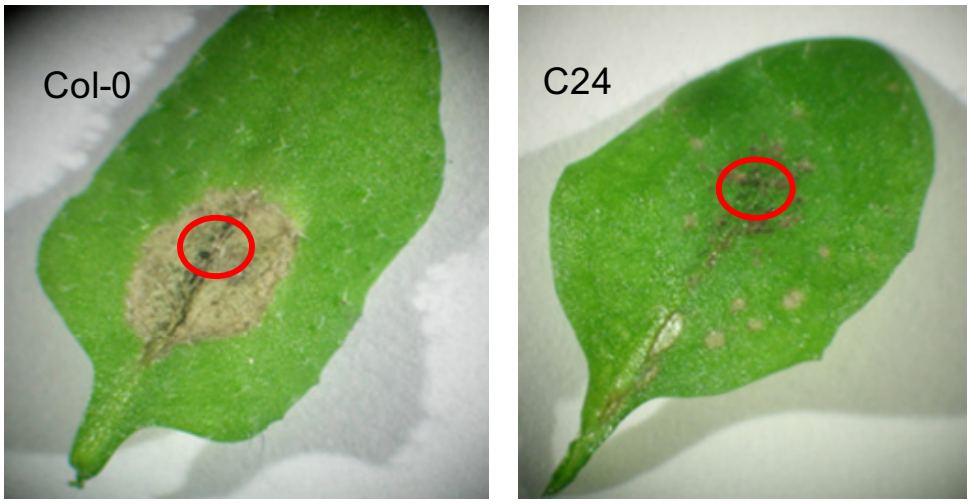

Figure 3.31 Necrotic local lesion formation in *A. brassicicola* -inoculated leaves of *A. thaliana* ecotypes Col-0 and C24. Red circles indicate where drops of *A. brassicicola* spores were applied.

3.3.5 Ecotypic variation in the gene expression between Col-0 and C24

Data mining on publicly available microarray data of several ecotypes was utilized to study the difference in gene expression between Col-0 and C24. These data were collected from the TAIR database (http://www.arabidopsis.org) and represents qc-RMA normalized microarray gene expression data from triplicates of aerial parts of 4 day old Col-0 and C24 seedlings grown in soil (Lempe, J. et al. 2005). 49 genes were found to be up-regulated two- to six-fold in C24 compared to Col-0 (see Appendix, Table 8.3. indicated in red). Amongst these 49 genes 22 were cell wall related based on their gene annotations from TAIR (Table 3.3. indicated in yellow). Most of the genes in this "cell wall" sub-group are hydrolases and extensins, as well as to a lesser extent apoplastic peroxidases. Interestingly, only one pectin related gene was identified in this expression comparison: a putative pectinase (*At4g02330*).

3.3.6 QTL analysis through RILs of Col-0xC24

In order to identify the genomic regions that determine the liquid culture phenotype difference of C24 to Col-0 and hence potentially the observed pectin phenotype, 209 recombinant inbred lines (RILs) were analyzed in liquid culture with pectinases. These RILs and the genotyping data were obtained from Prof. Thomas Altmann, representing the F10 progeny of a Col-0xC24 cross (Torjek, O. et al. 2006). The distribution of the SNP markers on the chromosomes was ubiquitous across all chromosomes (see appendix, Fig.8.4.)

The phenotypical data of the RILs were dissected into four quantifiable traits: hypocotyl length, cotyledon length, hypocotyl browning and meristem browning (see Fig.3.32). The visible phenotype of 3 individuals of each RIL was quantified and analyzed by image software and the retrieved data were analyzed by WinQTL software (see materials and methods 2.2.5.1.). The length was measured in centimeters and the browning was calculated as an index value of the ratio between red and green color when RGB values were obtained.

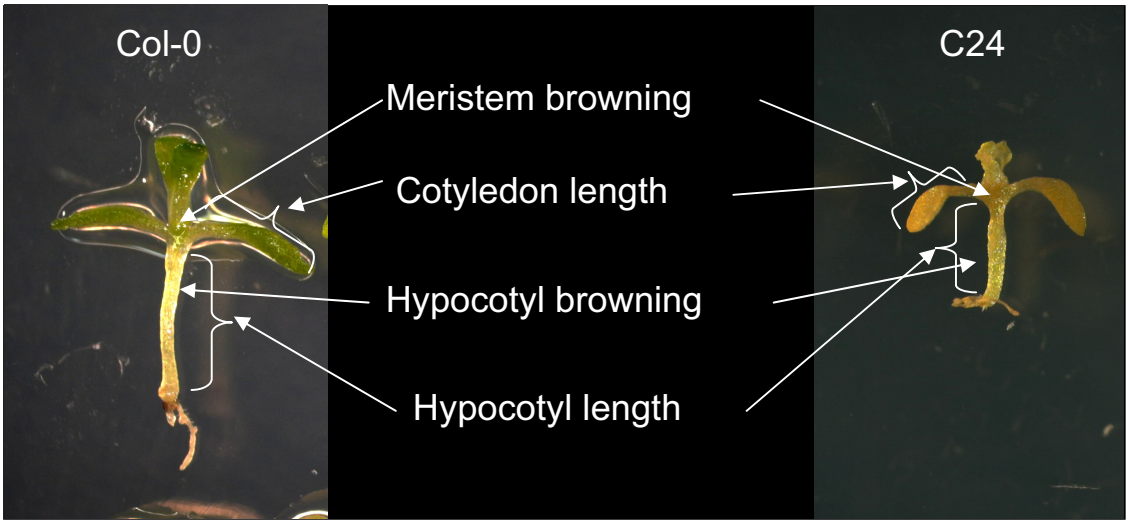

Figure 3.32 The four traits used for QTL analysis

A summary of the analyzed traits is given in Table 3.4. The midparent values (i.e. the values halfway between the two parents) and the median of the RILs were very similar for all the traits except the hypocotyl length, where the median was smaller than the midparent value (Table3.4.), therefore the population was slightly skewed to the left (Fig.3.35).

Table3.4 Mean and range values for quantitative traits of the RIL mapping population and their parents.

Trait	Parents		RILs		
	Col-0	C24	Minimum	Maximum	Median

Hypocotyl length	1.09 (±0.08)	0.71 (±0.01)	0.32	1.40	0.78
Colyledon length	0.87 (±0.10)	0.67 (±0.14)	0.35	1.20	0.68
Hypocotyl browning	1.43 (±0.04)	1.79 (±0.09)	1.31	2.24	1.66
Meristem browning	1.34 (±0.04)	2.02 (±0.19)	1.34	2.40	1.73

Correlation analysis showed a significant and strongly positive relationship between hypocotyl browning and meristem browning (correlation coefficient of 0.784 Table 3.5.). Hypocotyl length was moderately correlated with the cotyledon length. No significant correlation was observed between other traits (Table 3.5).

Table3.5 Pair-wise correlation coefficients of trait values of the RIL mapping population.

	Cotyledon length	Hypocotyl length	Hypocotyl browning
Meristem browning	-0.359	-0.269	0.784
Hypocotyl browning	-0.328	-0.296	
Hypocotyl length	0.415		

There was a continuous range of values for all measured traits among the RILs. Normal distributions were observed for all traits as illustrated by the histograms (Fig.3.33).

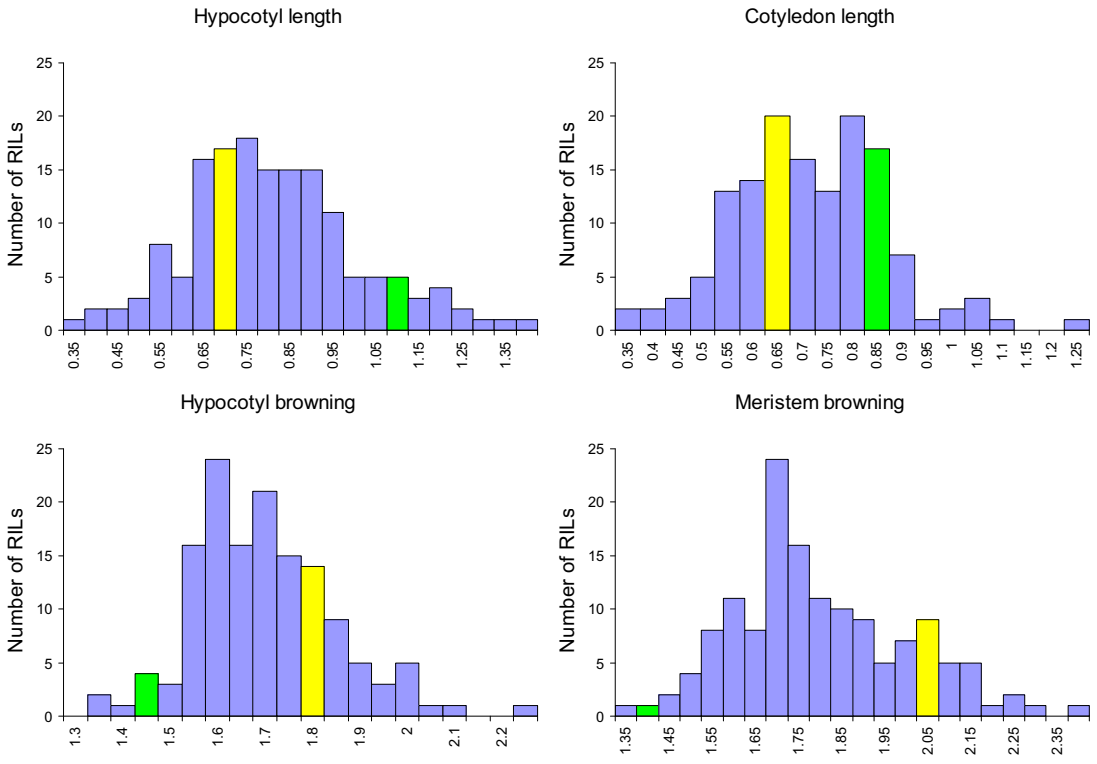

Figure 3.33 Distribution of trait values of the RIL population. Parental values are indicated by color. Green – Col-0, yellow – C24.

For QTL analysis simple interval mapping (IM) was employed (see 2.2.5.1.). This type of mapping method is an extension of single-marker analysis; it provides a systemic way to scan the whole genome for evidence of QTL (Zou, W. and Zeng, Z. B. 2008). IM uses two flanking markers to construct an interval within which to search for a QTL. Then, a LOD score (logarithm of odds) is calculated at each increment in the interval. Based on the IM analysis of this population, 3 QTLs could be identified: one on the right end of chromosome 1, where two QTLs partially overlapped (hypocotyl length and hypocotyl browning), one QTL on chromosome 3, where a close overlap was observed for the hypocotyl browning and meristem browning, and one on the left end of chromosome 5, corresponding to hypocotyl length. The trait cotyledon length did not produce a strong QTL, having a minor peak at the left end of chromosome 3 (Fig.3.34.). The two traits that were mostly correlated – hypocotyl browning and meristem browning (Table 3.5.) were also closely correlated in the IM results (Fig.3.34).

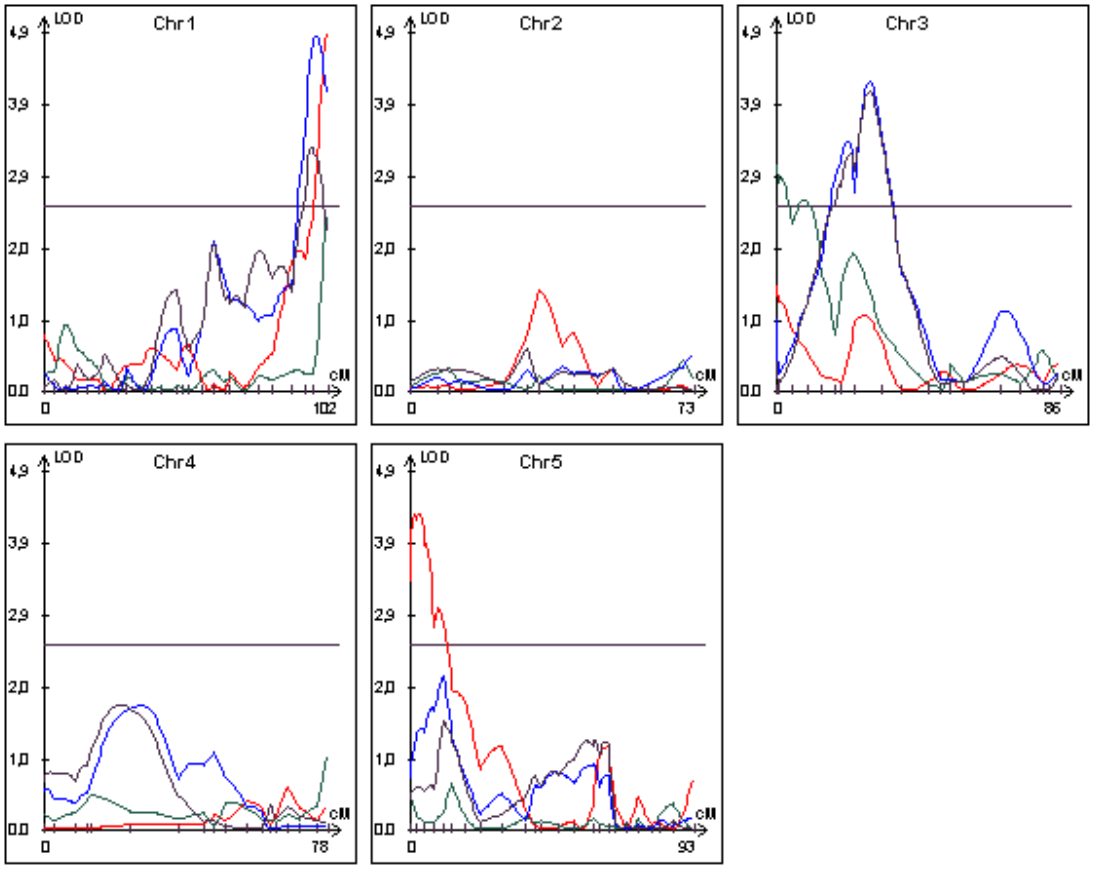

Figure 3.34 Interval QTL mapping of the 4 traits analyzed. Red – hypocotyl length, blue - hypocotyl browning, green – cotyledon length and black – meristem browning. LOD threshold is 2.5, abscissa is in cM.

Four QTLs for the three traits analyzed (hypocotyl length, hypocotyl browning and meristem browning) were further defined. The map location, log of the odds ratio (LOD) score, additive effect, and its standard error accounted for by each locus are given in Table 3.6. Two loci were associated with hypocotyl length and one locus on chromosome three was associated with both hypocotyl and meristem browning. The size of the genetic map interval for the QTLs ranged from 4 cM for QTL 4 to 14 cM for QTL 3 (Table 3.6).

Table 3.6 Summary of QTLs affecting the visible phenotype of liquid culture grown *A. thaliana* in presence of pectinases. Hyp. - hypocotyl, Mer. – meristem.

QTL	Chromosome	Map position (cM)		LOD	Trait	Additive effect	Std. error
1	1	94	102	4.62	hypocotyl browning	-0.060	0.012
1	1	98	102	4.94	hypocotyl length	0.072	0.015
2	3	20	34	4.13	meristem browning	0.082	0.017

3	5	0	4	4.24	hypocotyl length	-0.070	0.014

In conclusion, genetic mapping of the traits observed in liquid culture identified 4 QTLs ranging from 4cM to 14cM. These genetic regions are worth analyzing further to study the genetic basis of the observed differences between these two ecotypes in the pectinase containing liquid culture.

3.4 Reverse genetic screen of the PMT gene family in Arabidopsis

Recently, putative pectin methyl-transferase genes have been described (Mouille, G. et al. 2007). It is expected that this gene family is a potential target for modifying pectin methyl-esterification pattern, and hence pectin structure. A gene in this family, QUA2/TSD2 has already been shown to be involved in HG synthesis. Multiple lines of evidence support the designation of QUA2 as a putative pectin methyltransferase, although enzymatic evidence of function is still needed (Mouille, G. et al. 2007). To test if different members of this family fine-tune the pattern of methyl-esterification of HG, *in silico* analysis for potential candidates within this gene family was applied and a reverse genetic screen of T-DNA lines from these candidate genes for alterations in HG methyl-esterification pattern was performed. The T-DNA lines for these candidate genes were not tested in the established liquid culture with pectinases because they are *a priori* identified as potential mutant with altered pectin structure.

3.4.1 Characterization of the PMT gene family in Arabidopsis

This family contains a putative methyl transferase domain, annotated as DUF248 (domain of unknown function 248). The transmembrane hidden Markov model (TMHMM) prediction server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) predicted that all members of this family are type II transmembrane proteins that have a short single transmembrane helix at the N-terminus followed by and a long luminal C-terminus. The ARAMEMNON database (www.aramemnon.botanik.uni-koeln.de) predicts that these proteins are localized in the secretory pathway (appendix, table 8.8.). Most of this proteins were assigned as Golgi localized, based on a proteomics study of the *A. thaliana* organelles (Dunkley, T. P. et al. 2006). The length of the proteins is rather constant, containing on average 604 amino acids, with an average

Mw value of 68.7 kDa. The isoelectric point (pI) varies from 4.7 to 9.8, suggesting a plausible functionality at different pH.

The predicted topology of the PMTs indicates that the N-terminus is cytosolic and the catalytic domain resides in the endomembrane system, such as the Golgi lumen. A BLAST search for structural domains revealed the presence of an S-adenosyl-L-methionine (SAM) binding domain and a plant-specific putative methyltransferase domain (DUF248). The proposed enzymatic reaction and the domain structure of the PMT proteins are depicted in Fig.3.35.

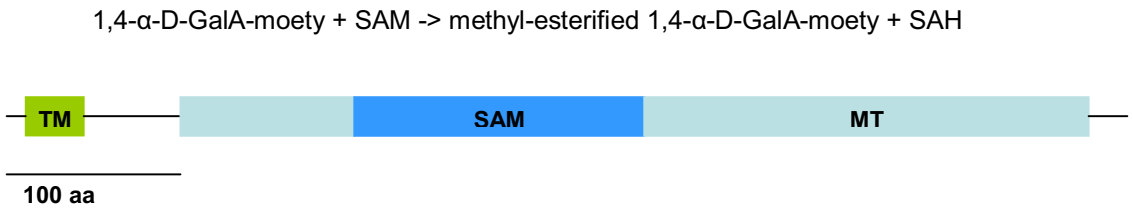

Figure 3.35 Schematic diagram of the enzymatic reaction and structure of the PMT proteins. The green box indicates the predicted TM domain, the blue box – the S-adenosyl-L-methionine (SAM) binding domain and the putative methyl transferase (MT) domain is shown in light blue (Krupkova, E. et al. 2007). The scale bar is in amino acids. SAH - S-adenosyl-L-homocysteine.

The gene family was constructed using BLAST search on the Arabidopsis protein database. This search revealed 34 genes homologous to qua2/tsd2. Phylogenetic analysis of this 35 protein sequences was done by using ClustalX (as described in 2.2.5.3.). This analysis showed that most members of this gene family originate from recent gene duplications as most of them are grouped in pairs (Fig. 3.36)

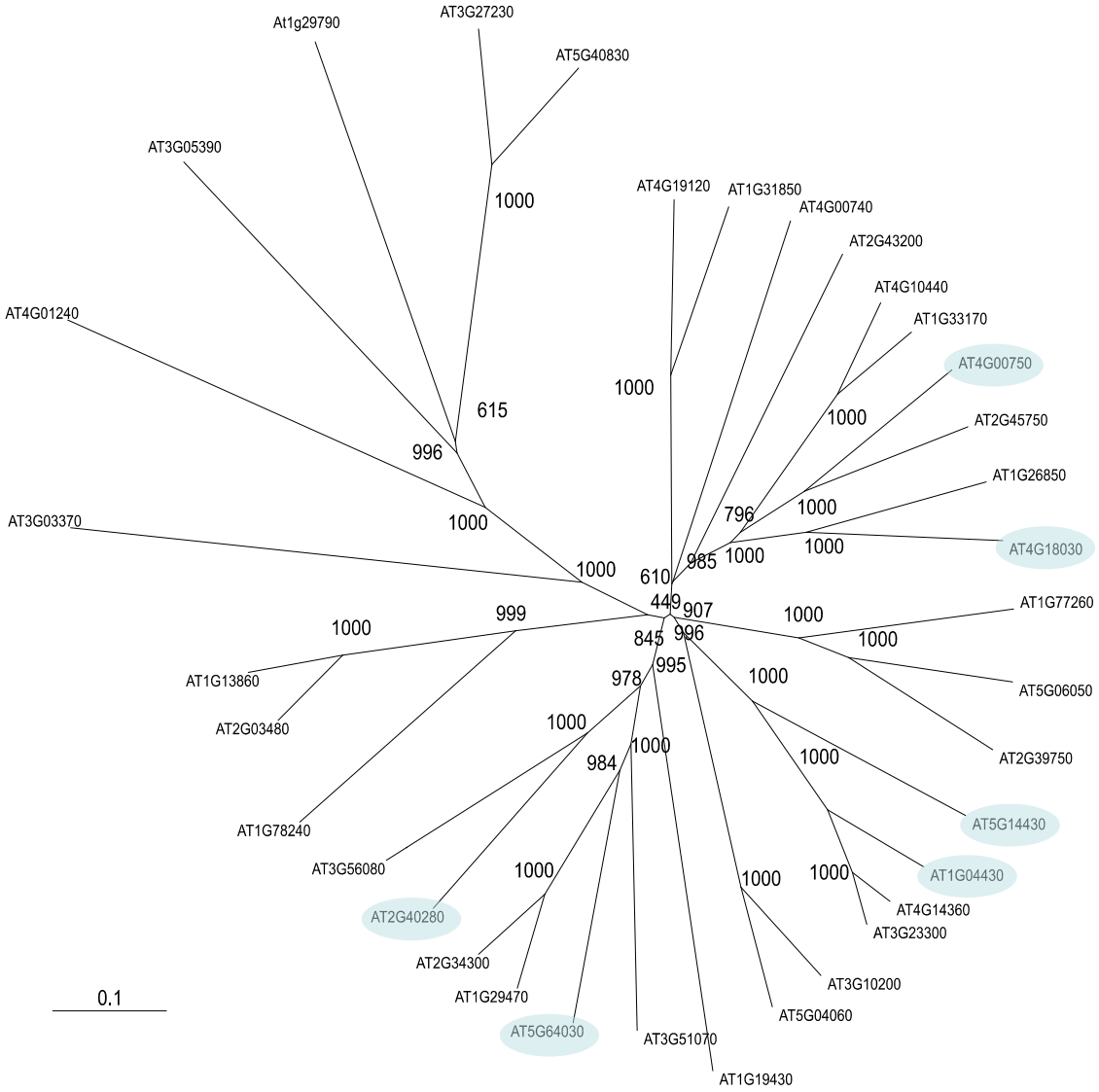

Figure 3.36. Phylogenetic consensus tree of *A. thaliana* PMT family generated from alignment of the predicted 35 full-length protein sequences. Amino acid sequences were analyzed by the neighbor-joining method using ClustalX2 (Thompson, J. D. et al. 2002). The length of the branches is proportional to the expected numbers of amino acids substitutions. One thousand cycles of bootstrap iterations were used and the bootstrap values are shown for each node. Genes annotated in blue are further described in the text.

3.4.1.1 Gene expression analysis

Gene-specific expression data for all members of the PMT gene family was collected from Genevestigator (Zimmermann, P. et al. 2005). These data are presented with a color code to provide absolute expression levels for comparison of the broad expression pattern over

different developmental stages. Gene specific expression data could be obtained for every PMT gene, except for the pair At1g13860 / At2g03480, for which cross reactivity of probes could be possible. Fig.8.5. in the appendix reports expression of the 33 PMT genes in 54 different organs of *A. thaliana* Col-0 WT. This data is clustered based on similarities in the gene expression pattern. This clustering illustrates examples of specialization of the PMT genes. For example, there are two genes that are expressed highly in the sperm cell and two in pollen and stamen, indicating on their potential specialized function in these cell types during their development and/or function (Fig.8.4 “sperm cell”, “pollen”, “stamen”). In contrast, the majority of genes have quite high and broad ubiquitous pattern of expression, some times having similar patterns of expression. The co-expression pattern however did not correlate to the phylogenetic relationship, and the gene pairs that were found homologous by their amino acid sequences did not show similar pattern of expression, therefore the role of this gene duplication may be in driving speciation of the duplicated genes.

In order to focus on some of the PMT candidates, co-expression analysis was performed. Gene co-expression analysis is a powerful approach for gene prioritization since in many cases implies the presence of a functional linkage between genes. All of the genes in the PMT family were used as bait for co-expression analysis using the ATTED II algorithm (www.atted.jp, (Obayashi, T. et al. 2009). This tool utilizes data from 58 microarray experiments (1388 slides) and searches for the closest co-expressed genes in the *A. thaliana* genome (Obayashi, T. et al. 2009). The output of this analysis is presented as a gene network (fig.3.37). This data shows the PMT genes that are closely co-expressed with already characterized or putatively involved genes in pectin biosynthesis including nucleotide sugar interconversion enzymes and HG galacturonosyl transferases. Other genes of unknown function also clustered with the PMTs, but since they could not point out the individual relevance of the PMTs to pectin biosynthesis, these data are not useful at this stage.

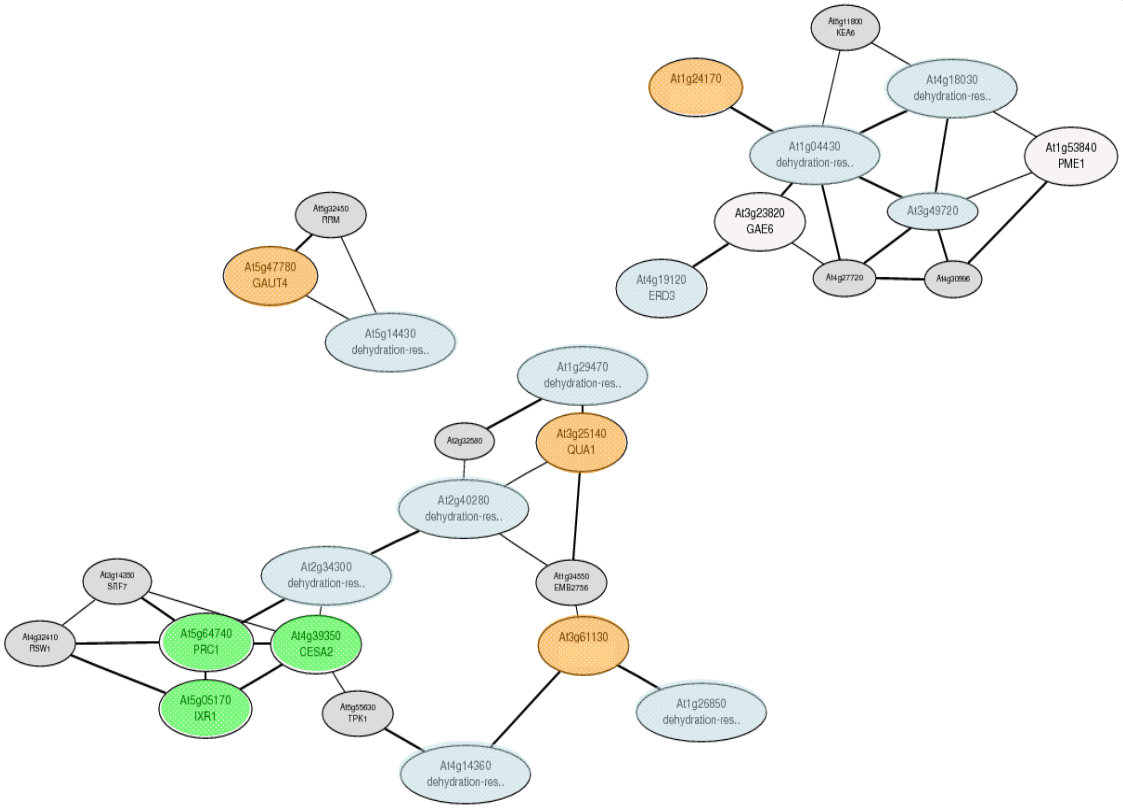

Figure 3.37 Coexpression analysis of the PMT genes. The network was constructed by the ATTED II tool (www.atted.jp) using PMT genes as bait. The blue ovals represent members of PMT family closely co-expressed with identified genes involved in pectin biosynthesis. Orange ovals represent characterized glycosyl transferases from the GT8 family involved in synthesis of HG. Green ovals represent genes of the CesA family involved in cellulose biosynthesis.

Table 3.8 List of selected genes from the PMT family.

Gene ID	Length (aa)	TM helix topology	Co-expression (ATTED II)	T-DNA lines
At1g04430	623	i13-35o	GATL8, GAE6 and SAM dependent MT	Flag_387E07 GK_863_E03
At2g40280	589	i7-26o	QUA1/GAUT8	SALK_098670C Flag_189H03
At4g00750	633	i21-43o	UDP-glucoronosyl/UDP-glucosyl transferase	SALK_97452 Flag_448D10 SAIL_744_C10
At4g18030	621	i13-35o	PME1, GATL8	SALK_047211C GABI_291A10
At5g14430	612	i16-38o	GAUT4	SALK_145020 SALK_03658
At5g64030	829	i17-39o	GAUT1	SALK_110446C

3.4.1.2. OLIMP for candidate genes

Based on this *in silico* analyses, six genes were selected for further studies (table 3.8.; Fig. 3.38). Homozygous T-DNA insertion lines for these genes were obtained from the WallNet project (http://www.uk.plbio.kvl.dk/plbio/wallnet/index.htm), an initiative for Functional Genomics of Plant Cell Wall Biogenesis. These lines were analyzed by oligosaccharide mass profiling (OLIMP) for oligogalacturonides released upon digestion with endoPG/PME (as described in 2.2.3.4). In brief, cell wall material from etiolated seedlings was treated with endoPG and PME and released oligogalacturonides were analyzed by MALDI-TOF (see materials and methods 2.2.3.12.). The analysis indicated that only the two T-DNA alleles for one gene, *At1g04430* displayed alterations in the OLIMP spectral analysis compared to the corresponding WTs (full data set: appendix, fig. 8.6). These data, represented in fig.3.38, point out that both T-DNA lines of *At1g04430* display changes in the oligogalacturonides' abundance; a decrease in the methyltrigalacturonide (GalA3Me) and a slight increase in the acetylmethyltrigalacturonide (GalA3Me1Ac). This indicates that a T-DNA insertional line of *At1g04430* has a perturbed pectin structure, thus representing a novel pectin mutant.

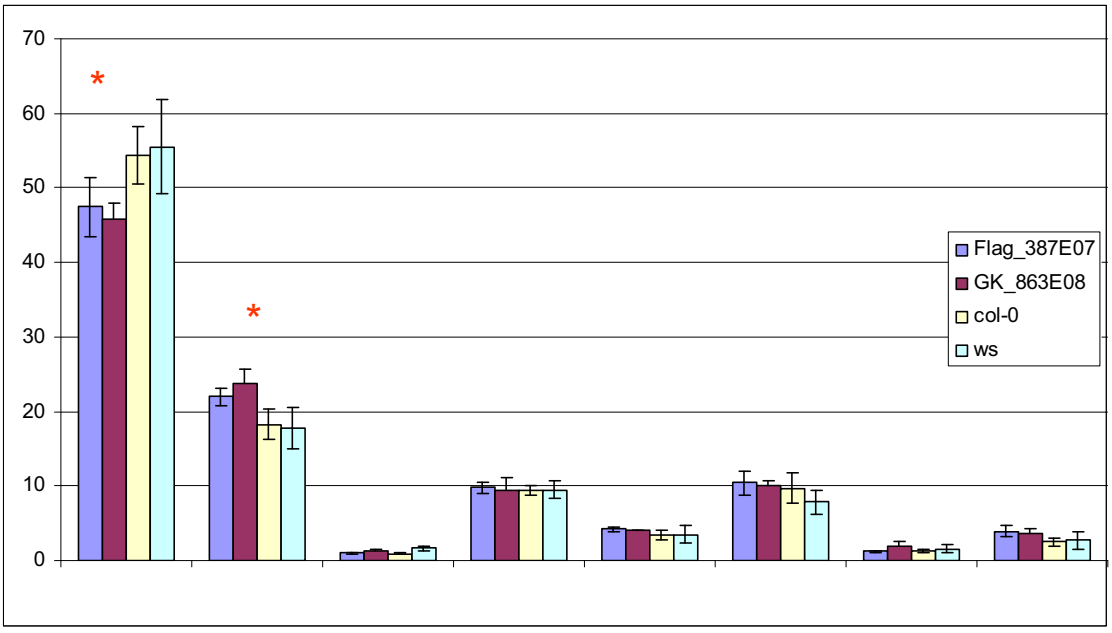

Figure 3.38 OLIMP of endoPG/PME digested cell wall material. The two T-DNA lines correspond to *At1g04430*. Each class represents different oligogalacturonides (OGAs). GalA – galacturonic acid Me – methyl ester, Ac acetyl ester, numbers indicate GalA monomers and esters. Asterisks indicate statistical difference from the WT ($p < 0.05$), error bars represent standard deviation, n=6.

In summary, this approach showed that at least one gene from the PMT family, besides QUA2/TSD2, may act as a methyltransferase by fine-tuning the pattern of methyl-esterification of HG. Future experiments will be necessary to determine the pectin structure of this mutant in detail, and to ascertain the enzymatic activity of the encoded protein to demonstrate its precise function.

4. Discussion

4.1. Establishment of a pectinase-based liquid culture screen of *A. thaliana*

Pectic polysaccharides are major components of primary cell walls and are amongst the most complex macromolecules in nature (Ridley, B. L. et al. 2001). Because of their structural complexity, the progress in understanding the role of distinct pectin structures on cellular processes has been slow. A major goal in pectin biology is to understand this structural-functional relationship. One way to investigate this relationship is by using genetic approaches, both forward and reverse.

Previous genetic screens for mutants altered in wall polysaccharide chemistry focused mainly on the monosaccharide profile obtained after acid hydrolysis or enzymatic digestion (Reiter, W. D. et al. 1997). These approaches only utilized analysis of the neutral monosaccharides, and not of the uronic acids, hence very few mutants affected in pectin structure were identified by them. Another consequence of this strategy is that a phenotypical scoring is done on the total monosaccharide composition of the cell wall, and not specifically on a certain cell wall polymer. This may prove to be misleading. For example, a mutant with quantitatively decreased levels of xylan, but with compensating levels of other xylose-containing polymers (such as xyloglucan or xylogalacturonan) might not be selected because the total xylose content would be unchanged. Therefore, to improve and facilitate selection for mutants that are affected in the deposition of a specific cell wall polymer (pectin) a new method of screening was developed.

A whole-organism medium-throughput pectinase-based screen was established, which enabled the screening of large pools of EMS and T-DNA insertional mutants as well as natural Arabidopsis variations. An important advantage of a whole-organism screen is that the treatment would provide a visual phenotype as an indicator of alteration in the pectin biosynthetic machinery or pectin-mediated signaling pathways. In order to confirm this and secure the correlation between the visible phenotypic alterations and the biochemical phenotype of the cell wall composition, the effect that this treatment had on the cell wall was thoroughly investigated on wild-type (WT) Arabidopsis individuals.

When WT *A. thaliana* plants are grown in the presence of fungal pectinases (endoPG/PM or PL alone) they show many growth and developmental abnormalities; they are delayed in development and display a “bleached” phenotype, compared to plants grown without enzyme (see 3.1 and Fig. 3.1). In nature, these pectinases are part of the battery of enzymes that pathogenic fungi secrete in order to deconstruct the plant cell wall and penetrate the cell. They specifically act on depolymerization of HG, thus breaking down the pectic network. When applied in liquid culture, these enzymes would act on whole organism level leading to cell separation by breaking down the middle lamellae which are almost exclusively composed of HG (see Fig.1.2). We expected to observe structural changes in the cell wall, mainly a decrease in the total levels of HG, and also a decrease in the methy-esterification of HG. In order to test this, detailed analyses of the changes that occur in the structure of the cell wall polysaccharides were performed. Complementary biochemical studies (cell wall monosaccharide composition, FTIR microspectroscopy and immunocytochemical analysis of HG) showed that the enzyme treatment had an effect on overall polysaccharide composition, especially on pectin structure. HG levels were decreased and elevated levels of RG-I side chains (arabinan and galactan) were recorded. This observation, namely an increase in RG-I chains following pectinase treatment was not expected and could be explained by an increase in cellulose-bound pectin, which in turn would generally reduce pectin digestibility. This hypothesis is also supported by the recent data showing that arabinan and galactan side chains of RG-I have a cross-linking function and are capable of covalently binding to cellulose (Zykwinska, A. et al. 2005).

It was also found that there is a correlation between the concentration of enzyme applied and the changes on cell wall polysaccharide level. Additionally, the observed decrease of uronic acid levels in total cell wall monosaccharide composition was also confirmed with FTIR microspectroscopy; a decrease of spectral bonds corresponding to unesterified, as well as methyl-esterified HG was observed. Immunohistochemical analysis with HG-directed mAb (JIM7) showed a reduced labeling in the cortex and epidermis of stems from plants grown in the presence of pectinases. All of these findings indicate a strong correlation between the observed visual phenotype and the cell wall composition, allowing for the development of a mutant screen where an altered visual phenotype (macrophenotype) would correspond to changes on the cell wall level (microphenotype). This link between the macro- and microphenotype was efficiently

employed to screen for putative pectin structural mutants on the basis of their macrophenotype, without the structural analysis of the microphenotype which is time consuming.

The established screening conditions were further tested on known cell wall-related mutants as a "proof of concept". In this study we were able to confirm that some of these characterized mutants showed alterations in the visible phenotype, further confirming the usefulness of this screening strategy.

4.2 Identification of novel pectin-related A. thaliana mutants through a pectinase based liquid culture screen

Our hypothesis is based on the assumption that, when grown in the presence of pectin-degrading enzymes, mutants with altered pectin structure would show an enhanced or dampened response compared to WT individuals, leading to a visibly altered plant phenotype. Mutant behavior may be explained by (1) an altered accessibility of pectinases to HG due to modifications in HG structure or in other cell wall components (especially the pattern of methyl-esterification), (2) a change in the abundance of the pectin network within the wall or (3) an alteration in the oligogalacturonide (OGA) signaling pathway. All of these processes could be impaired and selected for in the established screen. Therefore the types of mutants identified would fall in 2 categories: mutants with altered pectin structure (modification in pectin structure or abundance) and mutants challenged in pectin-mediated signaling. The later class could also display structural changes in pectin, but that is not always expected to be the case, since the mutants could be affected in the downstream signaling cascades after OGA elicitation.

Due to the setup of the screening procedure, the selection of false positive mutants (displaying a selection phenotype that is not due to the selection) could not be excluded. It is in fact reasonable to expect that some mutants could also show a visible phenotype due to other reasons such as impaired development. Furthermore, false positive mutants could also be selected due to the presence of true positives (displaying a selection phenotype that is due to the selection). For example, the release of OGAs from a mutant with aberrant HG structure could elicit developmental changes in other plants. To reduce the number of false positives, the procedure was designed to include a primary and a secondary screening step (see 3.2 and Fig. 3.9). Therefore, in this thesis, only data from mutant lines whose phenotype could be confirmed with both screening steps is discussed. Characterization of the selected mutants was prioritized

based on the severity of the visible phenotype (susceptibility represented by reduced growth, browning of the leaves, or cell separation).

4.2.1. Genetic screening of *A. thaliana* T-DNA tagged populations

In parallel to the identification and characterization of EMS-induced selected mutant lines, T-DNA insertional populations were also screened using the pectinase treatment. Genotyping of insertion lines on the molecular level is less time-consuming compared to the genotyping of chemically-induced mutants. The site of the insertion was identified using a PCR-based method (TAIL-PCR). During the primary screen, a total of approximately 10.000 individual T-DNA transformants were screened for an altered visual phenotype. Approximately 800 plants (~8% of all transformants) were selected and transferred to soil for seed amplification. Of those 800 lines, seeds were obtained from 708 lines. These lines were subjected to the secondary screen and 32 lines could be confirmed as true mutants (see table 3.1), corresponding to 4.5% of the selected lines from the primary screen. This low recovery rate could be due to the nature of the bulk selection in the primary screen; leading to a higher probability of selecting for false positive mutants. These lines were later genotyped by TAIL-PCR and the site of the insertion was determined for all of them.

The genes that resulted from the insertional analysis of the selected lines were categorized according to their putative function using Classification Superviewer (Provart, N. and Zhu, T. 2003). From this analysis it was concluded that the majority of the identified genes were in a functional category related to the cell wall, indicating that the established pectinase screen specifically selects for cell wall-related mutants. The rest of the genes range from transcription factors to metabolic enzymes; these genes are not likely to be directly involved in pectin biosynthesis, but are still interesting candidates that may regulate or modulate pectin metabolism. On a finer scale, the genes that were assigned as cell wall related can be subdivided in different categories. One of them is *PEC112*; this gene encodes a putative plasma membrane localized leucine rich repeat protein kinase that may be involved in perception of external signals, such as OGA signaling or sensing the cell wall integrity. *PEC117* is a putative apoplastic peroxidase involved in oxidative coupling of extracellular polymers. We have also identified two apoplastic proteases; *PEC105* encoding an aspartyl protease family protein and *PEC106* – a subtilase (subtilisin-like serine protease), presumably active in the cell wall. In this

list there is also a pectin modifying enzyme: *PEC131*, a pectin methyl-esterase which possibly modifies the methyl esterification of HG. There are also two cell wall degrading enzymes: *PEC114* (xylanase, hydrolyzing O-glycosyl compounds) and *PEC115* (xyloglucan:xyloglucosyl transferase). These genes, together with the genes putatively localized in subcellular compartments related to pectin biosynthesis and modification (the Golgi apparatus, the plasma membrane and the cell wall) encourage us to study the nature of their involvement in pectin biogenesis, metabolism and signaling.

We decided to focus our attention on one of them, *PEC100,* a putative non-nucleotide sugar transporter because, out of all the isolated genes, this was the only one that was predicted to encode for a secretory pathway-targeted protein. Considering that pectin biosynthesis is known to occur in the Golgi apparatus, we considered *PEC100* to be a promising candidate for further analysis.

4.2.1.1. PEC100 - a putative sugar transporter involved in pectin metabolism

The gene affected in *pec100-1* encodes a sugar transporter, presumably active in the secretory pathway. This gene is a member of a large family of transporters, named Major Facilitator Superfamily (MFS) and has a high homology to a lactose permease-related gene, indicating that this gene is most likely involved in sugar transport. The MFS is an ancient gene family, with members present in all living organisms: bacteria, archeobacteria, animals, plants and fungi (Pao, S. S. et al. 1998). MFS members include lactose permease from *E. coli*, glucose transporters in animals and hexose transporters in yeast.

A. thaliana has 161 MFS genes that are likely to encode sugar co-transporters. The MFS is a large superfamily of a diverse group of secondary transporters that includes uniport, symport and antiport proteins, with 12 or 14 transmembrane (TM) domains (Pao, S. S. et al. 1998). In *A. thaliana* and other plants, the sugar transporter protein (STP) subfamily (within the MFS) has been fairly well characterized. STP transporters are located on the plasma membrane and transport hexoses such as glucose, xylose, mannose, fructose, and galactose (Reinders, A. et al. 2005, Williams, L. E. et al. 2000). These transporters catalyze the proton-coupled uptake of hexoses (glucose, mannose, and galactose), sugar alcohols (sorbitol and inositol), pentoses (ribose, arabinose and xylose), and the tetrose sugar erythrose. Although *PEC100* is part of this

family, it still remains unclear whether it encodes a sugar transporter since its protein activity still needs to be verified.

PEC100 function was explored by transiently expressing the corresponding cDNA fused to the GFP open reading frame in tobacco. The protein fusion was shown to accumulate at the Golgi apparatus, thus confirming the targeting of PEC100 to the secretory pathway. This is a novel finding since the only kind of sugar transporters that have been shown to play a role in cell wall metabolism so far are nucleotide-sugar transporters that would allow for substrate efflux into the lumen of the Golgi to act as substrates for glycosyltransferases and glycansynthases (Reyes, F. and Orellana, A. 2008). So far several of these proteins have been described, namely GONST1 to GONST5, which are GDP-mannose transporters (Baldwin, TC. et al. 2001, Handford, M. G. et al. 2004) , AtNST-KT1 which transports UDP-galactose (Rollwitz, I. et al. 2006), AtUTr1 which transports UDP-galactose and UDP-glucose (Norambuena, L. et al. 2002), the UDP-galactose transporters AtUTr2, UDPGalT1 and UDPGalT2 (Bakker, H et al. 2005, Norambuena, L. et al. 2005). All of this enzymes apear to have distinct substrate specificity, and therefore cannot account for the transport of all substrates used for polysaccharide synthesis in the Golgi apparatus. Our data indicates that non-nucleotide sugar transporters such as PEC100 also play a role in cell wall biosynthesis, adding to the complexity of general cell wall metabolism. The biological mechanism by which PEC100 influences pectin structure still needs to be determined. One possibility is that this putative sugar transporter is involved in the import of mono- or disaccharides into the Golgi lumen and that inside the lumen these sugars could be phosphorylated by C-1 kinases to generate sugar 1-phosphates. In turn they themselves could act as substrates for the pyrophosphorylases that generate nucleotide sugars. However, based on this hypothesis these enzymes, together with the nucleotide sugar interconversion enzymes would have to be all Golgi-localized. Many of these interconversion enzymes (e.g., epimerases and dehydratases) appear to be membrane-bound and localized to the ER-Golgi apparatus (Burget, E. G. et al. 2003, Hayashi, T. et al. 1988). However, proof for the localization of C-1 kinases and pyrophosphorylases in the Golgi lumen still needs to be obtained. For example, most of the UDP-Glc pyrophosphorylase activity is cytosolic, although putative N-glycosylation sites, as well as biochemical data indicate the possibility of Golgi-associated isoforms (Eimert, K. et al. 1996). Another possibility is that this sugar transporter is indirectly involved in polysaccharide biosynthesis by contributing to the

necessary microenvironment in the Golgi apparatus (sugar concentration, viscosity, export of synthetic by-products from the Golgi) for proper Golgi functioning and polysaccharide synthesis.

The observed mutant phenotype was confirmed with a second allele, *pec100-2.* The specificity of the pectinase treatment-related mutant phenotype was confirmed by observing plant growth in the absence of pectinases and on soil. In both cases, no developmental alterations could be observed. *pec100-1* and *pec100-2* homozygous knock-out lines were further analyzed for any alterations in cell wall chemistry and showed a change related to RG-I, namely a decrease of cell wall-bound arabinose and rhamnose. Therefore, it is possible that this sugar transporter alters RG-I biosynthesis by changing substrate availability. Such influence has been shown for the nucleotide interconversion enzyme RHM2/MUM4, that has a mutation in the synthesis of UDP-rhamnose which reduces the content of rhamnose in pectin, thus influencing RG-I structure (Diet, A et al. 2006, Oka, T et al. 2007, Usadel, B et al. 2004). Recently it has been shown that an overexpression of the same gene increases the rhamnose content in the cell wall (Wang, J. et al. 2009). It is possible that the absence of or defects in the substrate transporters for pectin biosynthesis may perturbe the supply of substrates thus also affecting pectin composition. No mutants in Golgi localized nucleotide sugar transporters has yet been reported, therefore this hypothesis still remains to be tested (Reyes, F. and Orellana, A. 2008). Other plants with altered RG-I structure include transgenic lines that overexpress RG-I degrading enzymes and knock-out lines. For instance, a knock-out line for an alpha-L-arabinofuranosidase ARAF1 showed an increase in arabinan content but no visible phenotype (quite like PEC100), but its overexpressing line exhibited a delay in inflorescence emergence and altered stem architecture and increased xylan in secondary walls (Chavez Montes, R. A. et al. 2008). Another example includes the knock-out of ARAD1, a putative arabinosyltransferase that had a drastic effect on RG-I structure (arabinose content reduced to about 75%), but this had no effect on the cellular structure or development (Harholt, J. et al. 2006). These findings support our data, since we also observed that the alteration of RG-I in *pec100* had no effect on the visual phenotype, indicating that the functional plasticity of the cell wall polysaccharides may be responsible for the absence of a discernible visual phenotype when this mutant is grown on soil.

To further investigate the nature of the *PEC100* gene, its promoter activity was tested by following GUS gene expression by staining for protein deposition. Following transformation of WT *A. thaliana* Col0 plants with a *PEC100*promoter::GUS fusion construct, positive transformants were obtained that showed a high promoter activity in light grown seedlings which were uniformly stained. However, etiolated transformant seedlings were also analyzed with the same construct and showed a slight activity only at the elongation zone. These data indicate that *PEC100* is mainly expressed in tissue subject to primary cell wall synthesis (actively dividing cells) as shown in light-grown seedlings and is less involved in cell wall remodeling phenomena which characterize overall cell wall metabolism in etiolated seedlings.

Further studies on this sugar transporter will elucidate its substrate specificity. One may utilize the novel functional genomics approach of screening for transporter specificity by heterologous expression in *Xenopus* oocytes combined with sugar uptake assays (Nour-Eldin, H. H. et al. 2006). This platform has great potential for the assignment of plant transporter function and for identifying transporters for plant metabolites for which a transporter has not yet been identified (Nour-Eldin, H. H. et al. 2006).

4.3. Liquid culture screen of natural variations in *A. thaliana*

Our study of natural genetic variation in *A. thaliana* to pectinase treatment responsiveness under the designed liquid culture screening conditions revealed C24 as a susceptible *A. thaliana* ecotype. This ecotype was further investigated in comparison to Col-0 for differences in cell wall composition. Detailed analysis of HG revealed a change in methyl-esterification, coupled to a difference in the sensitivity to pectinases, both in liquid culture and in the ROS assay.

The finding that Col-0 and C24 accumulate HG at different levels and with differences in structural features was supported by the results from three independent analyses. First, monosaccharide composition of the crude cell wall material extracted from ecotype C24 showed a decrease in the levels of galacturonic acid. Second, there was a different pattern in the abundance of the oligogalacturonides seen by OLIMP for C24 compared to Col-0, reflecting a different pattern of HG methyl-esterification in C24. Third, C24 showed differential labeling when using HG-directed antibodies (JIM5 JIM7 and LM7) in comparison to Col-0. Therefore, we can conclude that these two ecotypes possess measurable differences in the HG domain of

pectin. Studies aimed at investigating C24 susceptibility to fungal pathogens showed that this ecotype is more responsive to the presence of fungal pectinases, since its ROS response was stronger compared to the one in Col-0. It has been shown that OGAs induce a transient accumulation of ROS, which by itself can act as a defense signal (see 1.4.1) and can be used as indicator for pectin mediated elicitation of plant defense responses (Bellincampi, D et al. 2000). Therefore, the increased accumulation of ROS induced by endoPG in C24 indicates a greater sensitivity to the enzyme in this ecotype. C24 also appears to be more resistant to the fungus *Alternaria brassicicola,* probably because of the increased sensitivity to fungal pectinases. Supporting data were obtained from publicly available microarray gene expression experiments (www.weigelworld.org/resources), showing that most of the genes that are 2-fold or more up-regulated in C24 are indeed cell wall-related, indicating that these two ecotypes probably have different cell wall properties.

Genetic mapping of the traits observed in liquid culture identified 3 QTLs (ranging from 4 to 14 cM) affecting the visual phenotype of *A. thaliana* in the presence of pectinases. Due to the nature of QTLs, the regions scored were rather large. The ultimate goal of the study of natural variation of the visible pectinase-based phenotype is to identify the exact nucleotide polymorphism that is responsible for each mapped phenotypic variation. Additional genetic resources can be utilized to make further progress. For example, it should be possible to map some of the QTLs to smaller regions using near-isogenic RILs (NILs) (Keurentjes, Joost J. B. et al. 2006). The NILs represent introgressions of chromosomal regions of one ecotype into the genetic background of another ecotype. Furthermore, with the recent advances in sequencing technologies, it is now possible to sequence large fragments of the *A. thaliana* genome in a relatively short amount of time. This so-called next-generation sequencing technology has enabled sequence analysis on a massive scale and opened new opportunities for polymorphism discovery in the Arabidopsis genome (Lister, R. et al. 2009). Several such methodologies have been developed. Massively parallel signature sequencing (MPSS), the first of the next-generation sequencing technologies, involved cloning cDNA fragments onto microbeads and a fluorescence-based sequencing method that generated a unique 17-base signature sequence for each mRNA at a specific site upstream from its poly(A) tail (Brenner, S. et al. 2000, Reinartz, J. et al. 2002). The original MPSS platform (Illumina, formerly Solexa) has since been replaced by an improved sequencing by synthesis (SBS) method, based on detecting the identity of a

nucleotide immediately after its incorporation into a growing strand of newly synthesized DNA. Other next-generation approaches include 454 Life Sciences' SBS pyrosequencing method, Applied Biosystems' SOLiD sequencing by ligation system, Helicos Biosciences' single-molecule synthesis platform, and several other single-molecule synthesis platforms that will be available soon (Gupta, P. K. 2008, Schuster, SC. 2008). These sequencing technologies are often referred to as ''deep sequencing'' because of their ability to generate huge numbers of sequencing reads per experiment or instrument run. In Arabidopsis, SBS pyrosequencing has been applied to two natural accessions to perform genome-wide SNP and InDel discovery (Ossowski, Stephan et al. 2008). This method is expected to provide unique opportunities to identify nucleotide polymorphisms between genetically closely related strains, such as ecotypes. Furthermore, the simultaneous genotyping thousands of SNPs on a genome-wide scale has also been recently developed (Steemers, F. J. and Gunderson, K. L. 2007). The combination of these two technologies will facilitated genetic dissection of complex traits by new methods for mapping QTLs, such as association mapping.

Association mapping, also known as linkage disequilibrium mapping, is a relatively new and promising genetic method for complex trait dissection. Association mapping has the promise of higher mapping resolution through exploitation of historical recombination events at the population level, that may enable gene level mapping on non-model organisms where linkage-based approaches would not be feasible (Nordborg, M. and Tavare, S. 2002, Risch, N. and Merikangas, K. 1996). Traditional linkage-based QTL mapping methods are often limited by the number of alleles sampled and their resolution, on the other hand association mapping is perfectly suited for sampling a wide range of alleles with high resolution (Flint-Garcia, SA. et al. 2003). Association mapping utilizes ancestral recombinations and natural genetic diversity within a population to dissect quantitative traits and is built on the basis of the linkage disequilibrium concept (Lewontin RC and Kojima K 1960). Linkage disequilibrium (LD) is the non-random co-segregation of alleles at two different loci. In contrast to linkage-based studies, LD-based genetic association studies offer a potentially powerful approach for mapping causal genes with modest effects (Hirschhorn, J. N. and Daly, M. J. 2005). While linkage analysis is based upon detection of non-random association between a genotype and a phenotype in well-characterized pedigrees, association mapping focuses on associations within populations of unrelated individuals. Essentially, association mapping exploits historical and evolutionary

recombination at the population level, rather than the recombination in inbred lines derived from a bi-parental cross (Yu, J. and Buckler, E. S. 2006). By exploring deeper population genealogy, association mapping offers three advantages over linkage analysis: much higher mapping resolution, greater allele number and broader reference population; and less research time in establishing an association (see Fig. 4.1) (Buckler, E. S. and Thornsberry, J. M. 2002, Flint-Garcia, SA. et al. 2003). As a result, highly defined polymorphism will be associated to a specific trait. The identified regions in this study are worth analyzing and further study by association mapping methods will probably reveal the genetic determinants (alleles and haplotypes) of the variation observed between these two ecotypes in the pectinase-based liquid culture assay. Therefore, it should be feasible to further study these candidate genes for a role in cell wall maintenance or pathogen sensing. This could be initiated by detailed studies of their expression patterns or by characterizing their biochemical function(s) using yeast-based heterologous expression systems. Genetic manipulations in terms of silencing or overexpression of the candidate genes could be used in order to determine that these genes are responsible for the observed phenotype. However, QTL analysis may reveal genes that are lethal if knocked out, since the causal allele or haplotype may differ in non-coding regions of the genes; in that case promoter manipulations may be employed.

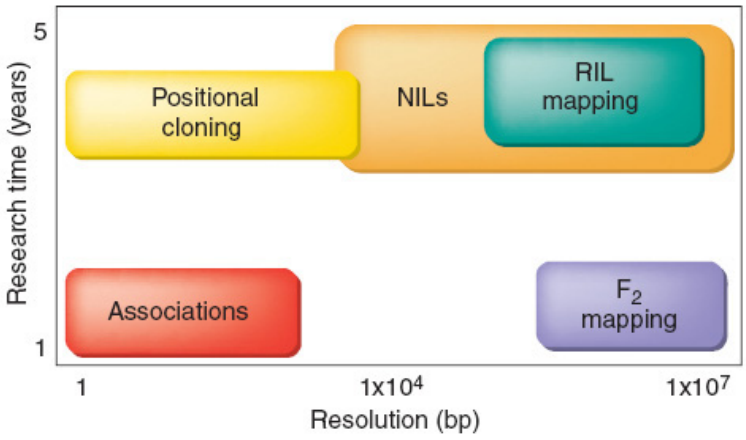

Figure 4.1 Comparison of resolution and research time for various approaches to dissect quantitative variation. The research times assume the target species has only two generations per year. NIL, near-isogenic line; RIL, recombinant inbred line (Buckler, E. S. and Thornsberry, J. M. 2002).

4.4. Reverse genetic screen of the PMT gene family in *A. thaliana*

In a reverse genetic screen, emphasis was placed on a pectin methyl-transferase (PMT) family as potential target genes that affect the pectin methyl-esterification pattern. A gene in this family, QUA2/TSD2 has already been shown to be involved in HG synthesis; mutations in this gene that disrupt protein function lead to a reduction in purifiable HG and to a drastic decrease in the methyl esterification of HG. Multiple lines of evidence support the designation of QUA2/TSD2 as a putative pectin methyltransferase, although enzymatic evidence of function is still needed (Mouille, G. et al. 2007). Pectin methyltransferase (PMT) activity from endomembrane preparations has been shown *in vitro* for several plant species (Bourlard, T. et al. 2001, Goubet, F. and Mohnen, D. 1999, Ishikawa, M. et al. 2000). These enzymes use S-adenosylmethionine (SAM) as a methyl donor, are membrane bound, and have a catalytic site located on the luminal side of the Golgi (Ridley, B. L. et al. 2001). The ability of UDP-GalA to stimulate PMT activity in intact membrane vesicles and of HG and pectin to serve as exogenous acceptors for PMT in detergent-permeabilized membranes, support a model in which a region of HG is synthesized before it is methylated by PMT (Mohnen, D. 2008).

Based on previous data, it has been proposed that there are two types of PMT activities depending on the pH, one with activity detected at pH 5.5 preferably active on low-methyl-esterified HG (DE 10%) and the second type with the highest activity at pH 7.0 and active on highly methyl-esterified HG (DE 50%) (Bourlard, T. et al. 2001). This is in line with the fact that the PMT family has quite a diverse isoelectric point (pI ranging from 4.7 to 9.8).

We focused on the hypothesis that different members of this family may fine-tune the pattern of methyl-esterification of HG. In order to test this, *in silico* analysis for potential candidates from this gene family was applied and a reverse genetic screen of T-DNA lines from these candidate genes for alterations in HG methyl-esterification pattern was performed. This approach showed that at least one gene from the PMT family, besides QUA2/TSD2, may act as a methyltransferase by fine-tuning the pattern of methyl-esterification of HG. The other 5 candidates tested did not show any detectable alterations in the OLIMP analysis, suggesting a functional redundancy for PMTs, tissue specificity or a possible activity on other polymers.

4.5. Overall impact

The research described in this thesis contributes to a better understanding of the pectin structural – functional relationship and biosynthesis. The finding that an altered pattern of homogalacturonan methyl-esterification correlates with susceptibility to fungal pectinases offers exciting opportunities to study the implication of pectin architecture in pathogen sensing. A better understanding of this process may ultimately lead to the development of crops with enhanced disease resistance by virtue of altering their pectin composition and structure. In view of the important role that fungal pectinases play in pathogenesis, it may be envisaged that plants with cell walls that are more resistant to pectinases will show a higher level of resistance to pathogens. Resistance to fungal pectinases may not reduce the occurrence of a primary infection but may slow down the lesion outgrowth. This may be sufficient to enable the host plant to activate other types of defense mechanisms. Furthermore, the QTLs identified offer the perspective of isolating genes that may be exploited to generate pathogen-resistant plants.

In regards to pectin biosynthesis, a novel putative non nucleotide-sugar transporter was found to be involved (directly or indirectly) in this process. This observation offers new ways of envisioning how sugar substrates could be imported/exported to the Golgi apparatus and suggests that polysaccharide synthesis may rely on different kinds of sugar transporters.

5 Summary

Pectic polysaccharides, a class of plant cell wall polymers, form one of the most complex networks known in nature. Despite their complex structure and their importance in plant biology, little is known about the molecular mechanism of their biosynthesis, modification, and turnover, particularly their structure-function relationship.

One way to gain insight into pectin metabolism is the identification of mutants with an altered pectin structure. Those were obtained by a recently developed pectinase-based genetic screen. *Arabidopsis thaliana* seedlings grown in liquid medium containing pectinase solutions exhibited particular phenotypes: they were dwarfed and slightly chlorotic. However, when genetically different *A. thaliana* seed populations (random T-DNA insertional populations as well as EMS-mutagenized populations and natural variations) were subjected to this treatment, individuals were identified that exhibit a different visible phenotype compared to wild type or other ecotypes and may thus contain a different pectin structure (*pec*-mutants).

After confirming that the altered phenotype occurs only when the pectinase is present, the EMS mutants were subjected to a detailed cell wall analysis with particular emphasis on pectins. This suite of mutants identified in this study is a valuable resource for further analysis on how the pectin network is regulated, synthesized and modified.

Flanking sequences of some of the T-DNA lines have pointed toward several interesting genes, one of which is *PEC100*. This gene encodes a putative sugar transporter gene, which, based on our data, is implicated in rhamnogalacturonan-I synthesis. The subcellular localization of PEC100 was studied by GFP fusion and this protein was found to be localized to the Golgi apparatus, the organelle where pectin biosynthesis occurs.

Arabidopsis ecotype C24 was identified as a susceptible one when grown with pectinases in liquid culture and had a different oligogalacturonide mass profile when compared to ecotype Col-0. Pectic oligosaccharides have been postulated to be signal molecules involved in plant pathogen defense mechanisms. Indeed, C24 showed elevated accumulation of reactive oxygen species upon pectinase elicitation and had altered response to the pathogen *Alternaria brassicicola* in comparison to Col-0. Using a recombinant inbred line population three major QTLs were identified to be responsible for the susceptibility of C24 to pectinases.

In a reverse genetic approach members of the *qua2* (putative pectin methyltransferase) family were tested for potential target genes that affect pectin methyl-esterification. The list of these genes was determined by *in silico* study of the pattern of expression and co-expression of all 34 members of this family resulting in 6 candidate genes. For only for one of the 6 analyzed genes a difference in the oligogalacturonide mass profile was observed in the corresponding knock-out lines, confirming the hypothesis that the methyl-esterification pattern of pectin is fine tuned by members of this gene family.

This study of pectic polysaccharides through forward and reverse genetic screens gave new insight into how pectin structure is regulated and modified, and how these modifications could influence pectin mediated signalling and pathogenicity.

6 Zusammenfassung

Pektin Polysaccharide, eine Klasse pflanzlicher Zellwand Polymere, formen eine der komplexesten natürlichen Strukturen. Trotz seiner immensen Bedeutung in der Biologie der Pflanzen sind die Kenntisse über die molekularen Mechanismen der Pektin Biosynthese, dessen Modifikation und Abbau überraschend gering.

Eine Möglichkeit neue Einblicke in den pflanzlichen Pektin Metabolismus zu erhalten, ist die Identifizierung von Mutanten mit veränderter Pektinstruktur. Solche Mutanten konnten durch ein neuatiges Selektionsverfahren gefunden werden. Zieht man Keimlinge der Ackerschmalwand (Arabidopsis thaliana) in Flüssigmedium mit Pektinase an, so lässt sich ein typischer Phänotyp beobachten: Die Pflanzen sind kleinwüchsig und leicht chlorotisch. Diesem Verfahren wurden Populationen verschiedener Genotypen (Insertions Linien, EMS Mutanten, natürlich vorkommende Varianten) ausgesetzt. Auf diese Weise wurden Individuen identifiziert, die gegenüber der Pektinase Behandlung eine verminderte oder erhöhte Resistenz aufweisen, was auf eine veränderte Pektinstruktur hindeutet.

Die EMS Mutanten wurden einer detaillierten Zellwand Analyse unterzogen. die so in dieser Arbeit identifizierte Kollektion von Mutanten stellt eine wertvolle Ressource für weitere Forschungsansätze zur Regulation, Biosynthese und Modifikation des Pektins dar.

Die Lokalisation der Insertionen in den T-DNA Linien führte zur Identifikation interessanter Gene, zu denen der putative Zuckertransporter PEC100 gehört. Dieses Gen steht vermutlich in Verbindung mit der Synthese von Rhamnogalakturonan-I, einem Bestandteil des Pektins. In dieser Arbeit konnte PEC100 im Golgi Apparat, dem Ort der Pektin Biosynthese, lokalisiert werden.

Die natürlich vorkommende Variante C24 ist besonders empfindlich gegenüber der Pektinase. Diese Empfindlichkeit konnte anhand rekombinanter Inzucht Linien auf drei bedeutende quantitative Merkmalsloci (QTL) eingegrenzt werden. C24 zeigte zudem ein gegenüber der Referenz verändertes Massenprofil der Oligogalakturonide. Diese werden derzeit als Signalmoleküle in der pflanzlichen Pathogenabwehr diskutiert, was mit der in dieser Arbeit geseigten Resistenz von C24 gegenüber Schwarzfleckigkeit verursachende Pilz (Alternaria brassicicola) korreliert.

In einem revers-genetischen Ansatz wurden zudem Mitglieder der Pektin Methyltransferase Familie als potentielle Enzyme getestet, die die Pektin Methylesterifikation beeinflussen könnten. Diese Mutation in einer dieser Methyltransferasen führte zu Veränderungen des Oligogalakturonid Massenprofils. Dies bestätigt die Hypothese, dass Mitglieder dieser Genfamilie an der Regulation der Methylesterifikation von Pektin beteiligt sind.

Die vorliegende Studie, in der ein genetishen Selektionverfahren und Methoden der reversen Genetik kombiniert wurden, hat neue Einblicke in die Regulation und Modifikation von Pektin geliefert.

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8 Appendix

8.1 T-DNA genotyping

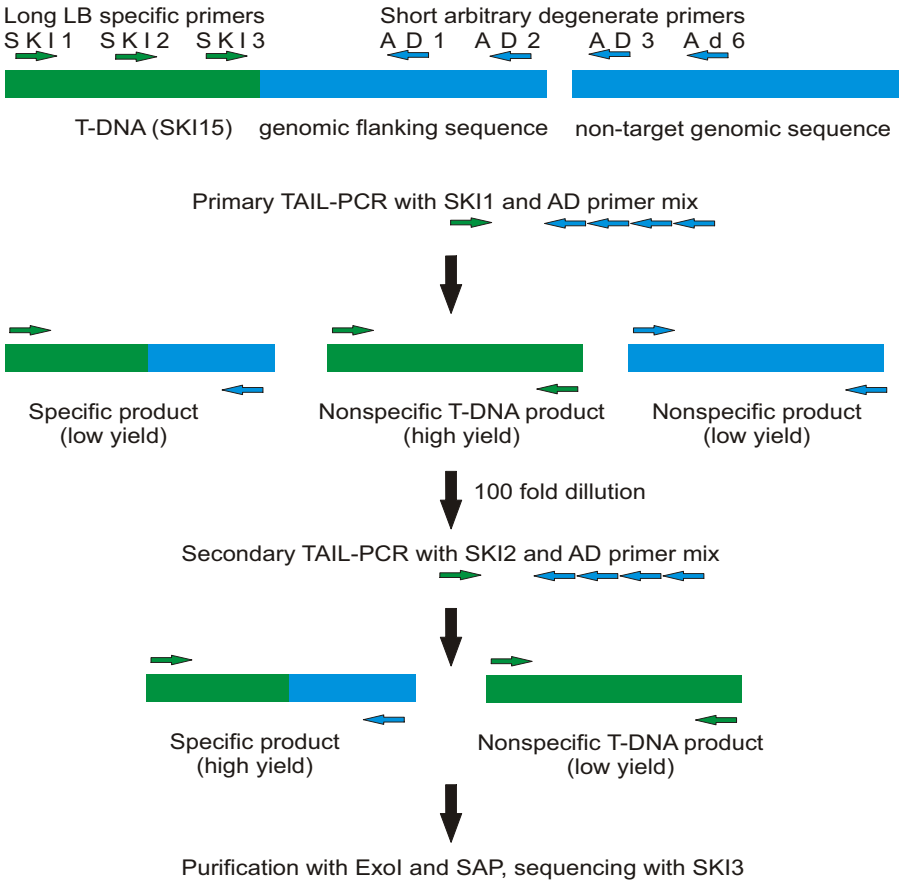


Figure 8.1. Strategy of T-DNA genotyping by using modified thermal asymmetric interlaced (mTAIL) PCR (Sessions A et al., 2002). Genomic DNA of selected lines is used as a template for the primary TAIL-PCR using a T-DNA specific primer (SKI1) and a mixture of arbitrary degenerate (AD) primers. The product of this reaction is used as a template for the secondary TAIL-PCR with the T-DNA specific primer (SKI2) and a mixture of AD primers. The product of this reaction is purified with exonuclease I (ExoI) and shrimp alkaline phosphatase (SAP) and submitted for sequencing with SKI3 primer.

Table 8.1 Summary of the T-DNA screened pools and the lines selected.

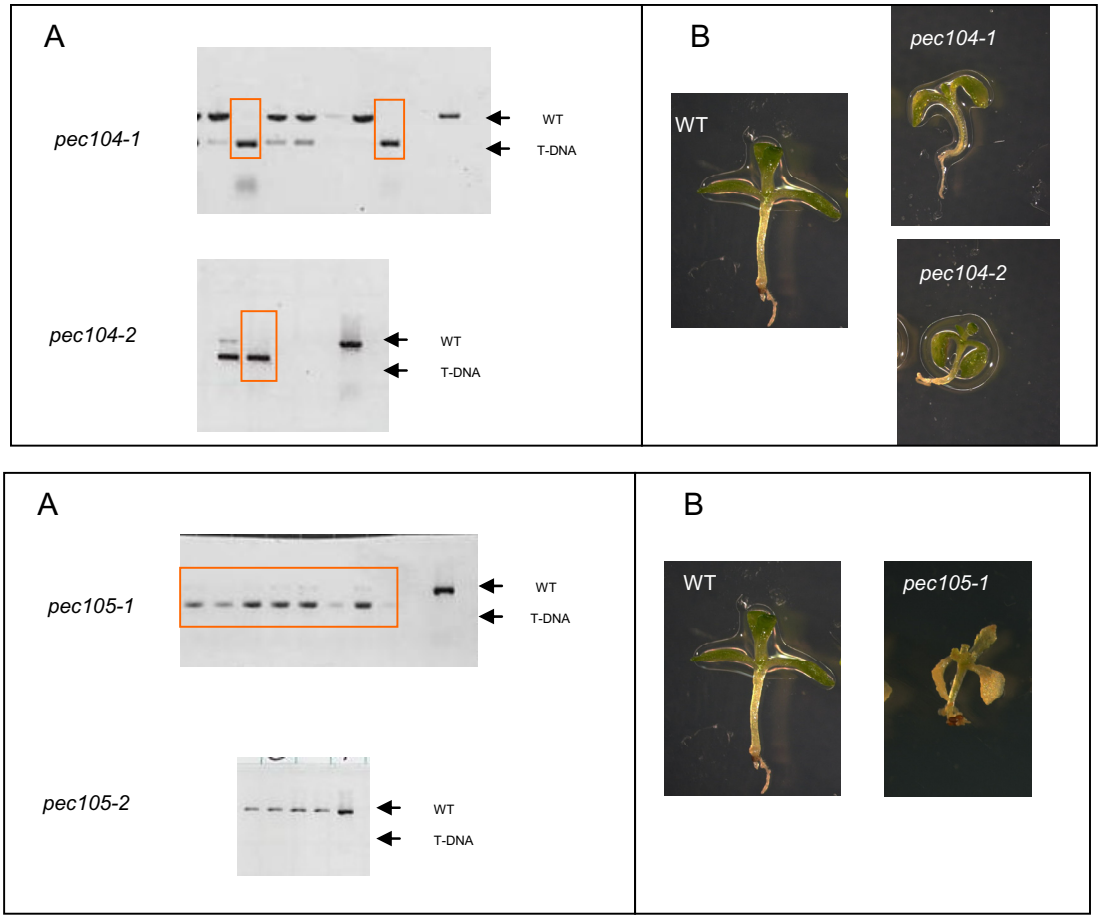
# of screens	Pools	# of lines analyzed in the secondary screen	# of confirmed mutants
1	T1-10A	272	5
2	T20A	22	2
3	T21A	59	1
4	T22A	58	3
5	T23A	74	5
6	T24A	19	3
7	T25A	23	9
8	T26A	23	1
9	T28A	29	1
10	T29A	16	0
11	T30A	28	3
12	T32A	25	1
13	T33A	5	0
14	T34A	20	0
15	T35A	16	0
16	T36A	17	1
17	T38A	2	0
	Sum	708	35

Table 8.2 List of identified T-DNA lines

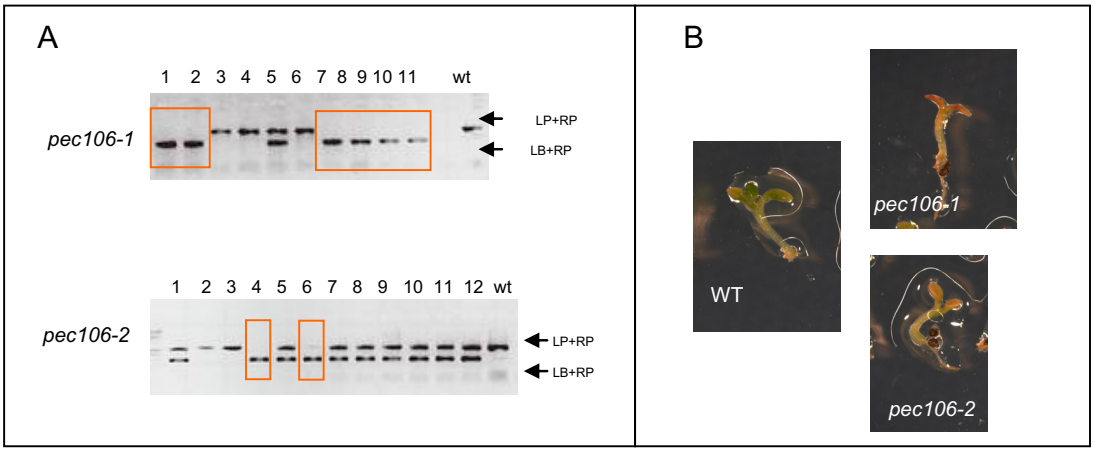
pec#	Gene ID	annotation	allele	Arbitrary name	Insertion site	allele status
pec100	At2g44280	lactose permease	pec100-1	2A42	gene	HoZ
			pec100-2	SALK_139158	gene	HoZ
pec104	At2g4000	Hs1pro-1	pec104-1	10A11	gene	HoZ
			pec104-2	SALK_016065C	gene	HoZ
pec105	At3g61820	aspartil protease	pec105-1	20A26		HoZ
			pec105-2	SALK_093873C		Only WT
pec106	At1g76740	unknown cell wall-anchored subtilisin-like serine protease	pec106-1	20A34		HoZ
			pec106-2	SALK_020245C		HoZ
pec107	At4g31680	transcriptional factor B3 family protein	pec107-1	21A33		HoZ
			pec107-2	SALK_055346	Exon	Hoz
pec108	At2g31800	ankyrin protein kinase	pec108-1	22A4		HoZ
			pec108-2	SALK_083275	Exon	HoZ
pec110	At5g09360	LAC14, laccase, multicopper oxidase	pec110-1	22A82		HoZ
			pec110-3	SALK_105129C		HoZ
pec111	At3g14225	GLIP4, carboxylic ester hydrolase, lipid metabolic process	pec111-1	23A30		HoZ
			pec111-3	SALK_048818	Intron	HoZ
pec112	At5g40910	disease resistance protein (TIR-NBS-LRR class)	pec112-1	23A36		HoZ
			pec112-2	SALK_043422C		HoZ
pec113	At1g24320	alpha-glucosidase, putative; similar to GCS1 GH63	pec113-1	23A40		Hoz
			pec113-2	SALK_137471C		HoZ
pec114	At1g58370	RXF12 xylanase	pec114-1	23A64		HoZ
			pec114-2	SALK_138469C		HoZ
pec115	At1g11545	xyloglucan	pec115-1	23A81		HoZ

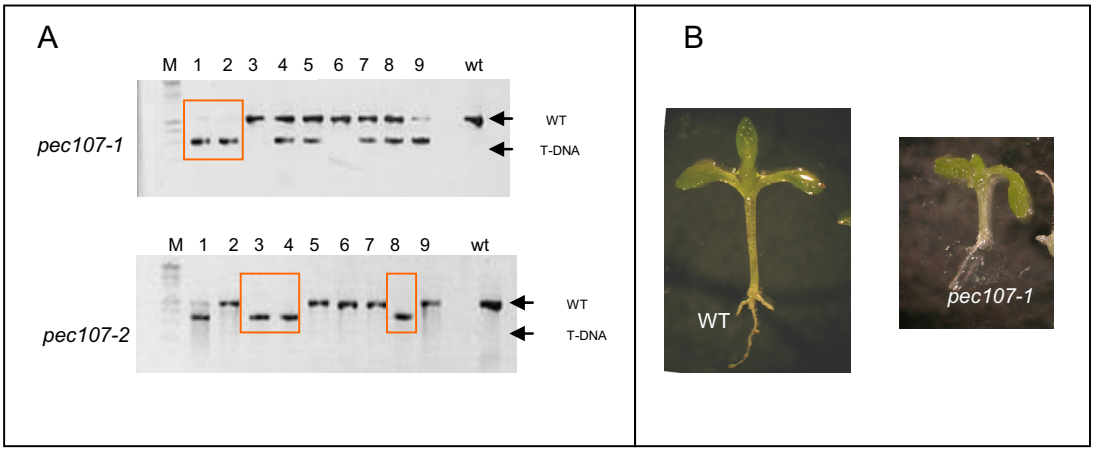
pec#	Gene ID	annotation	allele	Arbitrary name	Insertion site	allele status
		endotransglucosylase/hydrolase protein 8	pec115-3	SALK_150293	Intron	HoZ
pec116	At1g08010	zinc finger (GATA type) family protein (TF)	pec116-1	25A6		Hoz
			pec116-2	SALK_009203		Only WT
pec117	At1g05240	peroxidase, putative	pec117-1	24A9		HoZ
			pec117-2	SALK_103597C		HoZ
pec118	At5G09280	Pectate lyase family protein	pec118-1	24A15		HoZ
			pec118-3	SALK_025828C		Only WT
pec120	AT5G01030	Transcriptional factor tubby, unknown protein	pec120-1	25A8	gene	HoZ
			pec120-2	SALK_138105		HoZ
			pec120-3	SALK_102648C		HoZ
pec121	At1g09950	transcription factor-related; similar to ZW2	pec121-1	25A16	gene	Hoz
			pec121-2	SM_3_39561	Exon	Hoz
pec122	AT4G35830	aconitate hydratase, cytoplasmic / citrate hydro-lyase / aconitase (ACO)	pec122-1	25A19	5' UTR	HoZ
			pec122-2	SALK_026148		HoZ
pec123	At5g40470	LRR protein, similar to F-box family protein	pec123-1	25A21	gene	HoZ
			pec123-2	SALK_059174		HoZ
pec124	AT1G50110	branched-chain amino acid aminotransferase	pec124-1	25A25	double insertion	Hoz
			pec124-2	SALK_119480C		HoZ
pec126	At5g25150	WD-40 TAF5 (TBP-ASSOCIATED FACTOR 5)	pec126-1	25A25	double insertion	HoZ
			pec126-2	SALK_021380		Only WT
pec127	At4g35170	unknown protein, harpin induced	pec127-1	25A26	gene	HoZ
			pec127-2	SALK_100046C		Only WT
pec128	At5g14890	NHL repeat-containing protein, Soluble quinoprotein glucose dehydrogenase	pec128-1	26A34	gene	HoZ
			pec128-2	SALK_102818C		HoZ
pec129	AT1G26240	prp extensin	pec129-1	28A20	gene	Hoz
			pec129-2	SAIL_535_B04		HoZ
			pec129-3	WiscDsLox259H01		HoZ
pec130	At3g59830	ankyrin protein kinase	pec130-1	30A13	gene	HoZ
			pec130-2	SALK_130077C		Only WT
pec131	At5g18990	PME, pectinesterase family protein	pec131-1	30A26	gene	HoZ
			pec131-2	SALK_057847C		HoZ
			pec131-3	SALK_138949		HoZ
pec132	At2g39440	unknown protein PIG-P	pec132-1	30A27	gene	HoZ
			pec132-2	SALK_043851C		HoZ
pec133	AT1G15980	ADP-heptose:LPS heptosyltransferase	pec133-1	32A29		Hoz
			pec133-2	SALK_137420		Only WT

Fig.8.2. Identification of *pec* alleles. A. T-DNA genotyping. Red rectangle indicates homozygous (HoZ) T-DNA line, where only the T-DNA specific product (LB+RP primer pair) and no WT product (LP+RP primer pair) were present. B. Plants grown in liquid culture with 0.25 U/ml endoPG/PME.

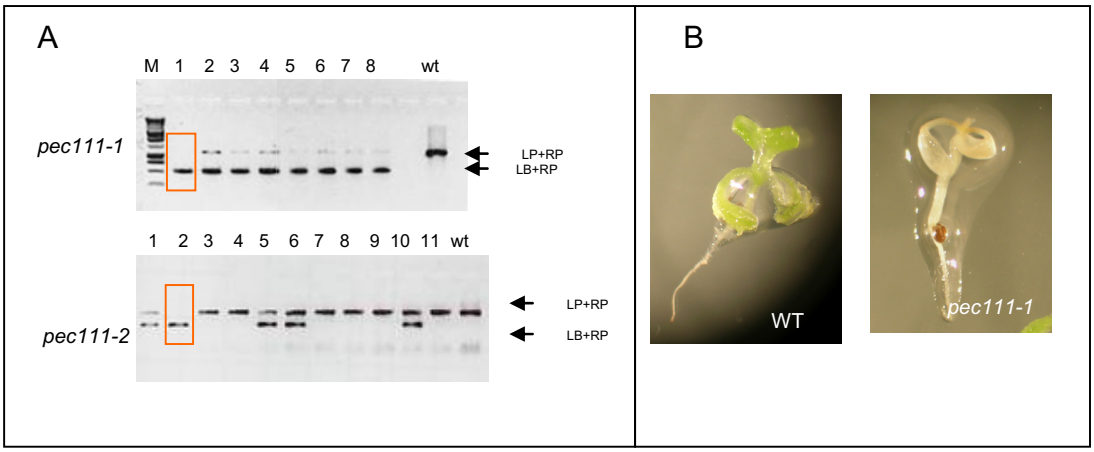


The second allele (*pec130-2,* Salk_130077C) was not found.

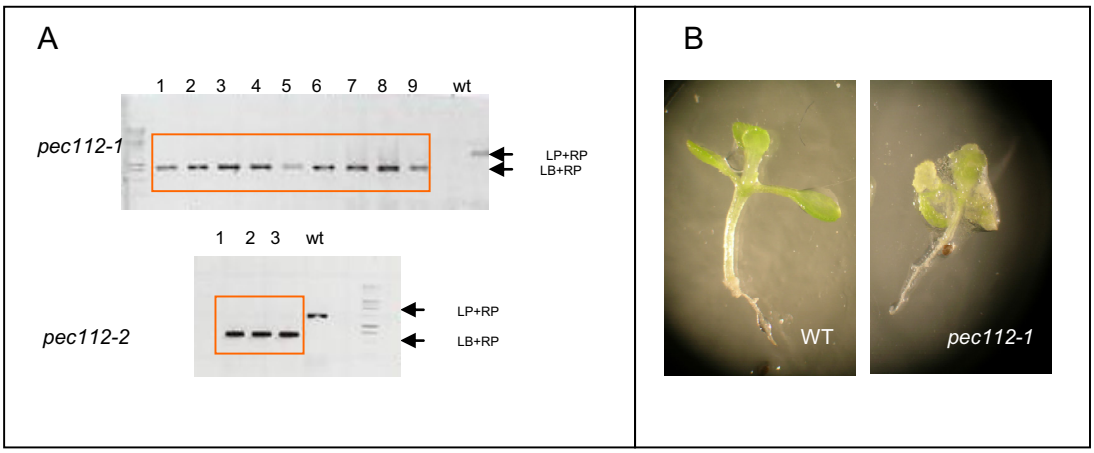




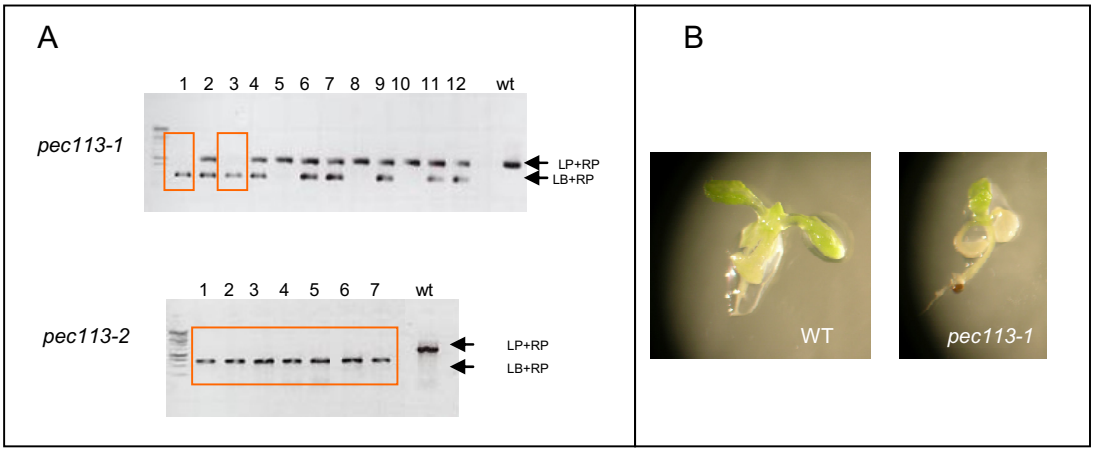
The second allele (*pec107-2*) has not been tested in liquid culture.



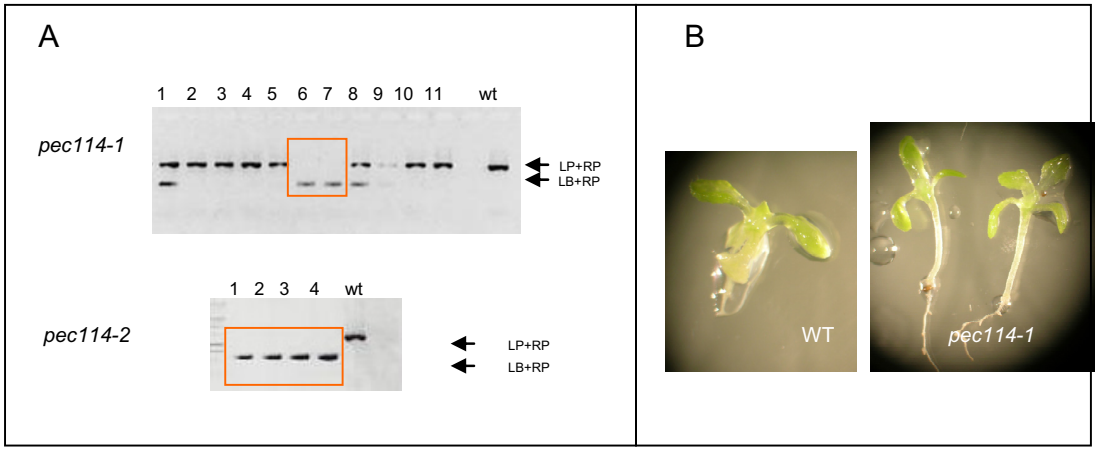
The second allele (*pec111-2*) has not been tested in liquid culture.



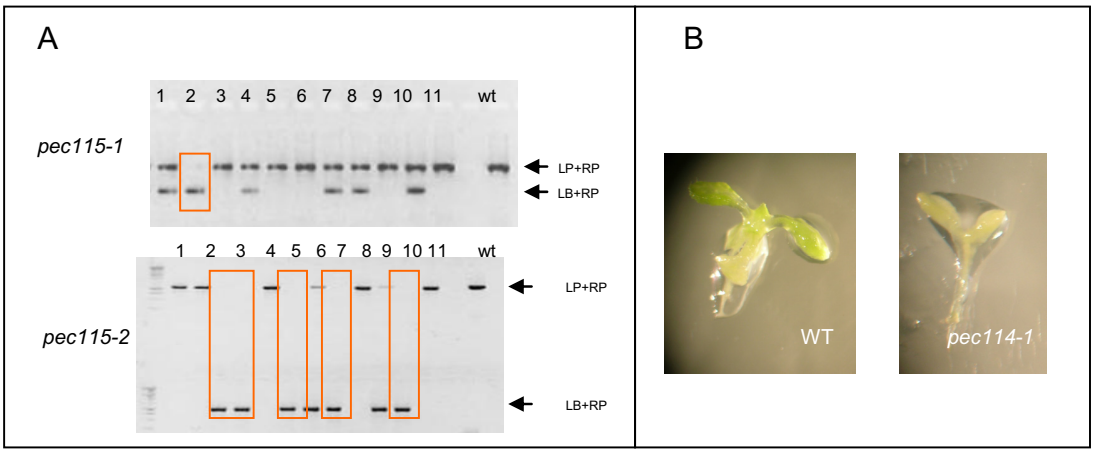
The second allele (*pec112-2*) has not been tested in liquid culture.



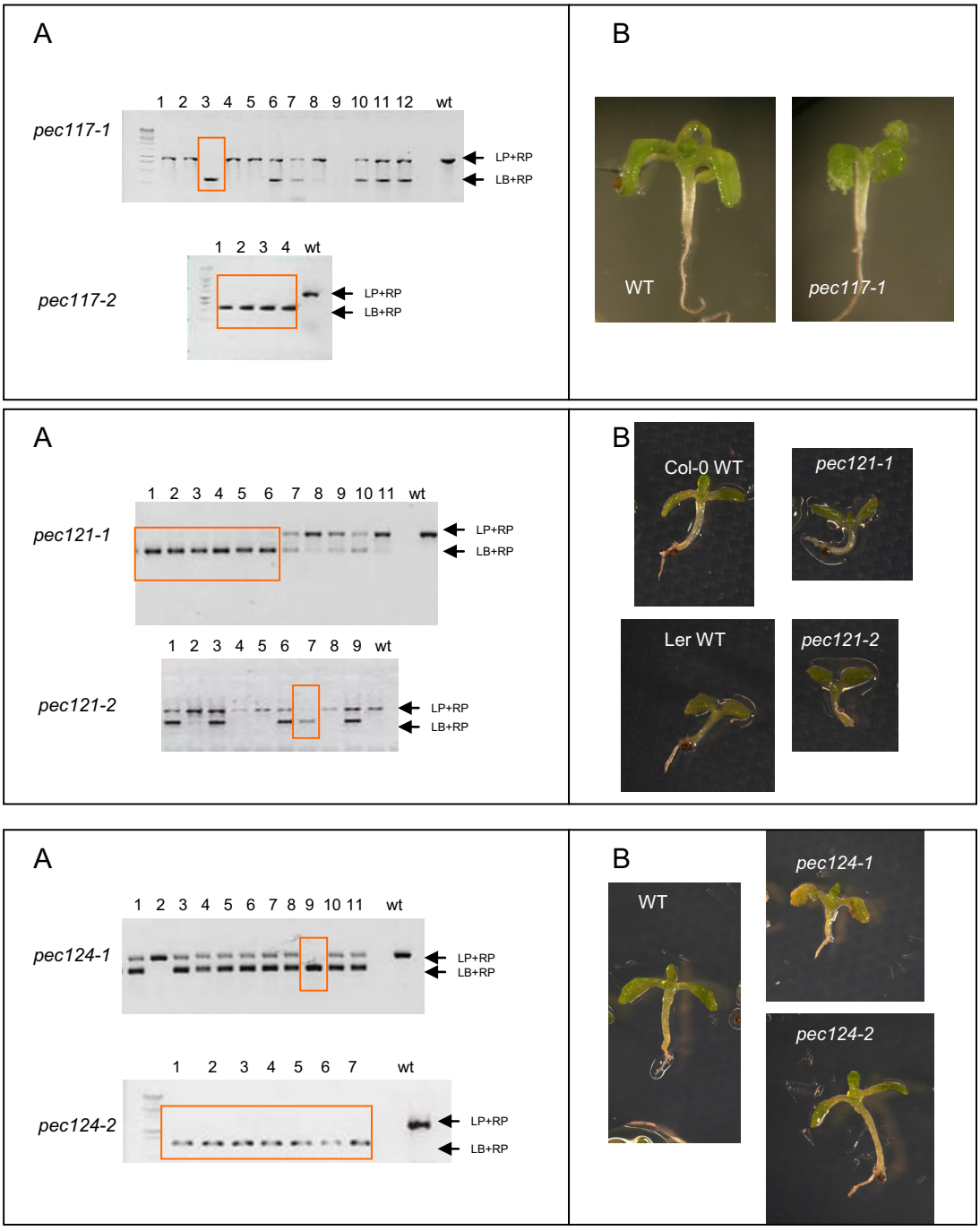
The second allele (*pec113-2*) has not been tested in liquid culture.

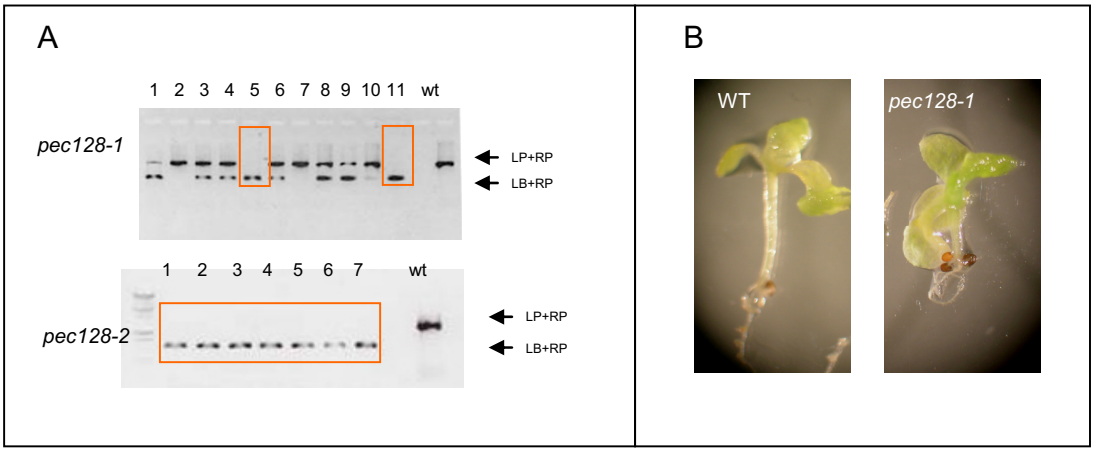


The second allele (*pec114-2*) has not been tested in liquid culture.

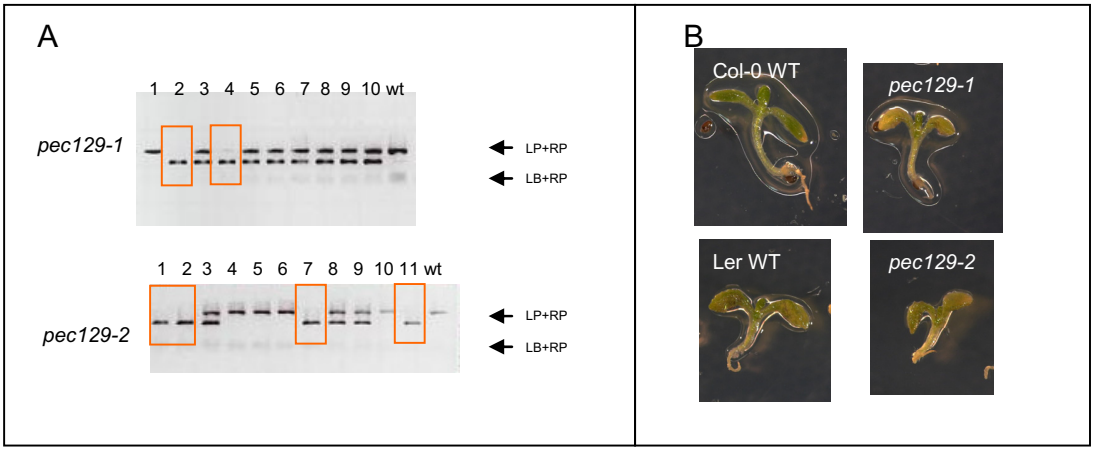


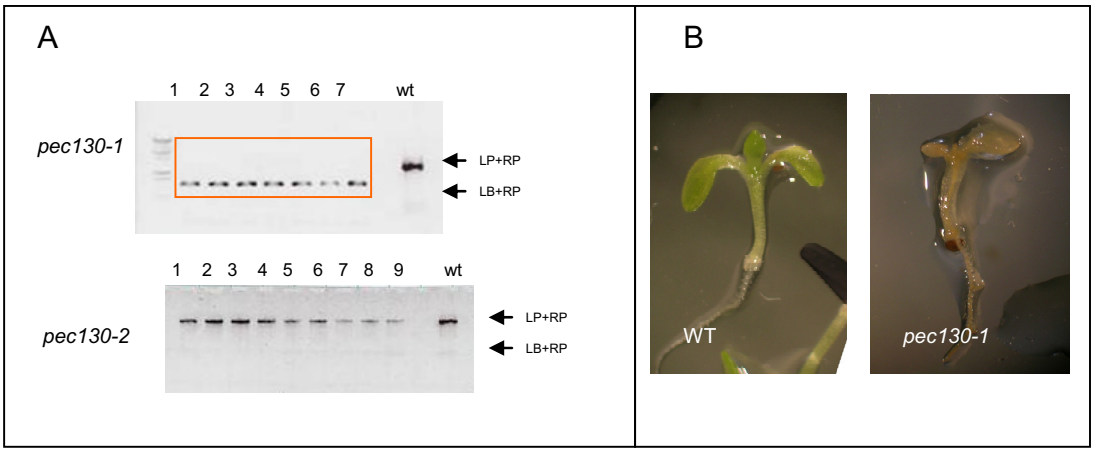
The second allele (*pec115-2*) has not been tested in liquid culture.


A
pec117-1
1 2 3 4 5 6 7 8 9 10 11 12 wt
LP+RP
LB+RP
pec117-2
1 2 3 4 wt
LP+RP
LB+RP
B
WT
pec117-1
A
pec121-1
1 2 3 4 5 6 7 8 9 10 11 wt
LP+RP
LB+RP
pec121-2
1 2 3 4 5 6 7 8 9 wt
LP+RP
LB+RP
B
Col-0 WT
pec121-1
Ler WT
pec121-2
A
pec124-1
1 2 3 4 5 6 7 8 9 10 11 wt
LP+RP
LB+RP
pec124-2
1 2 3 4 5 6 7 wt
LP+RP
LB+RP
B
WT
pec124-1
pec124-2

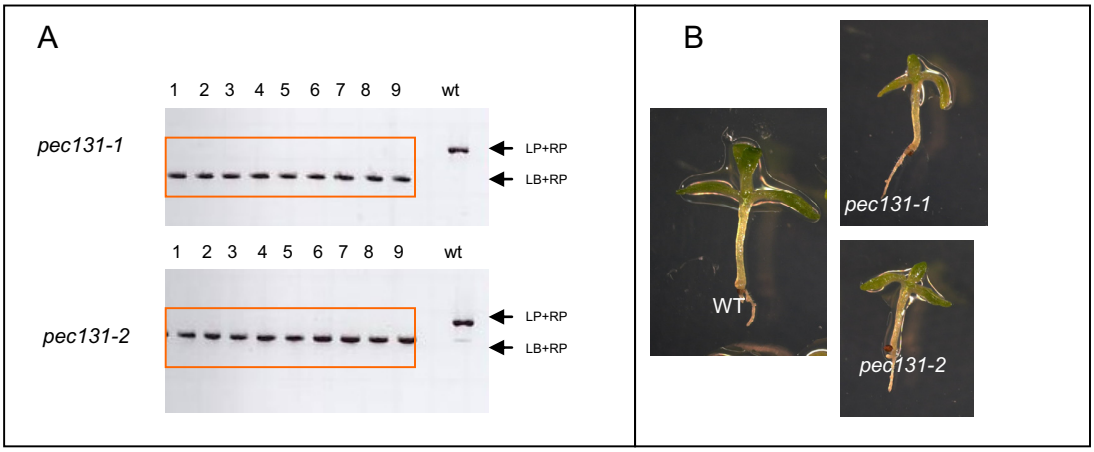


The second allele (*pec128-2*) has not been tested in liquid culture.

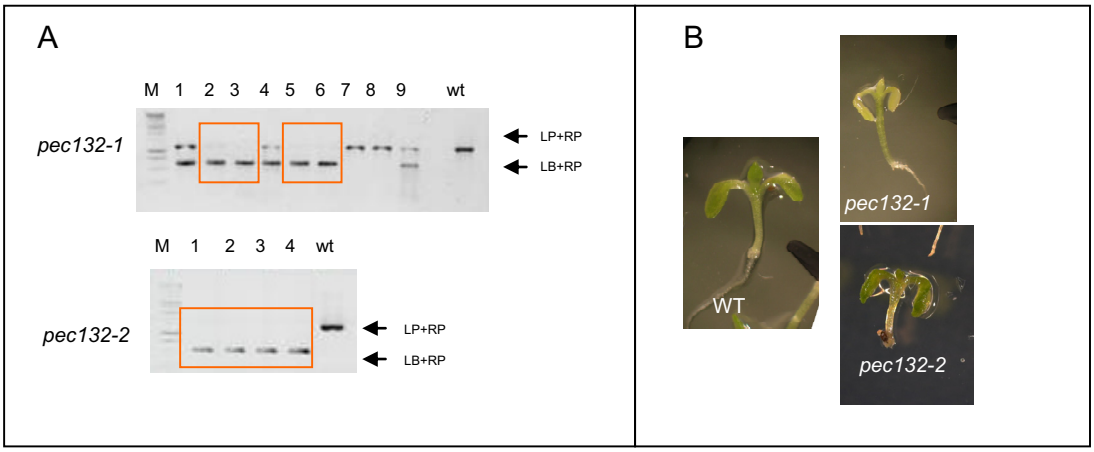




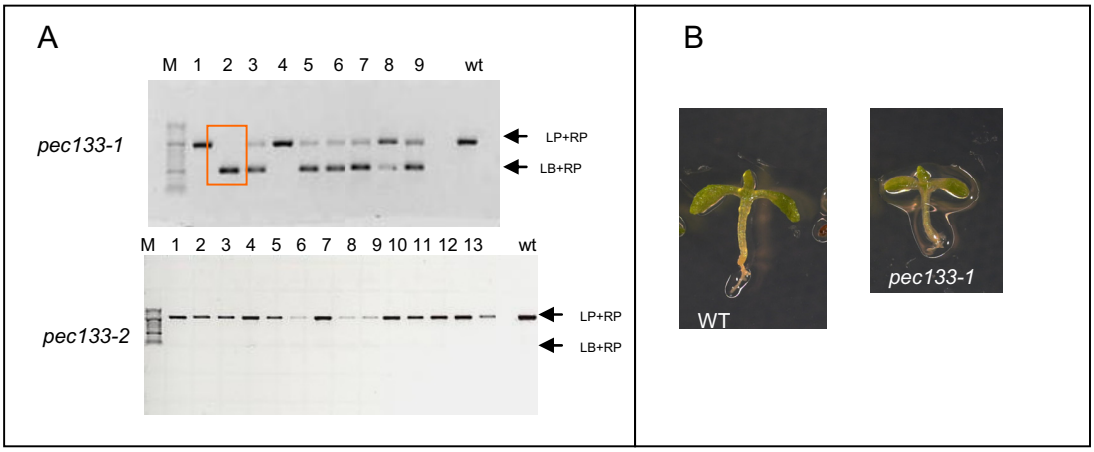
The second allele (*pec130-2,* Salk_130077C) was not found.



The second allele (*pec131-2*, Salk_ 057847C) did not reproduce the initial phenotype.



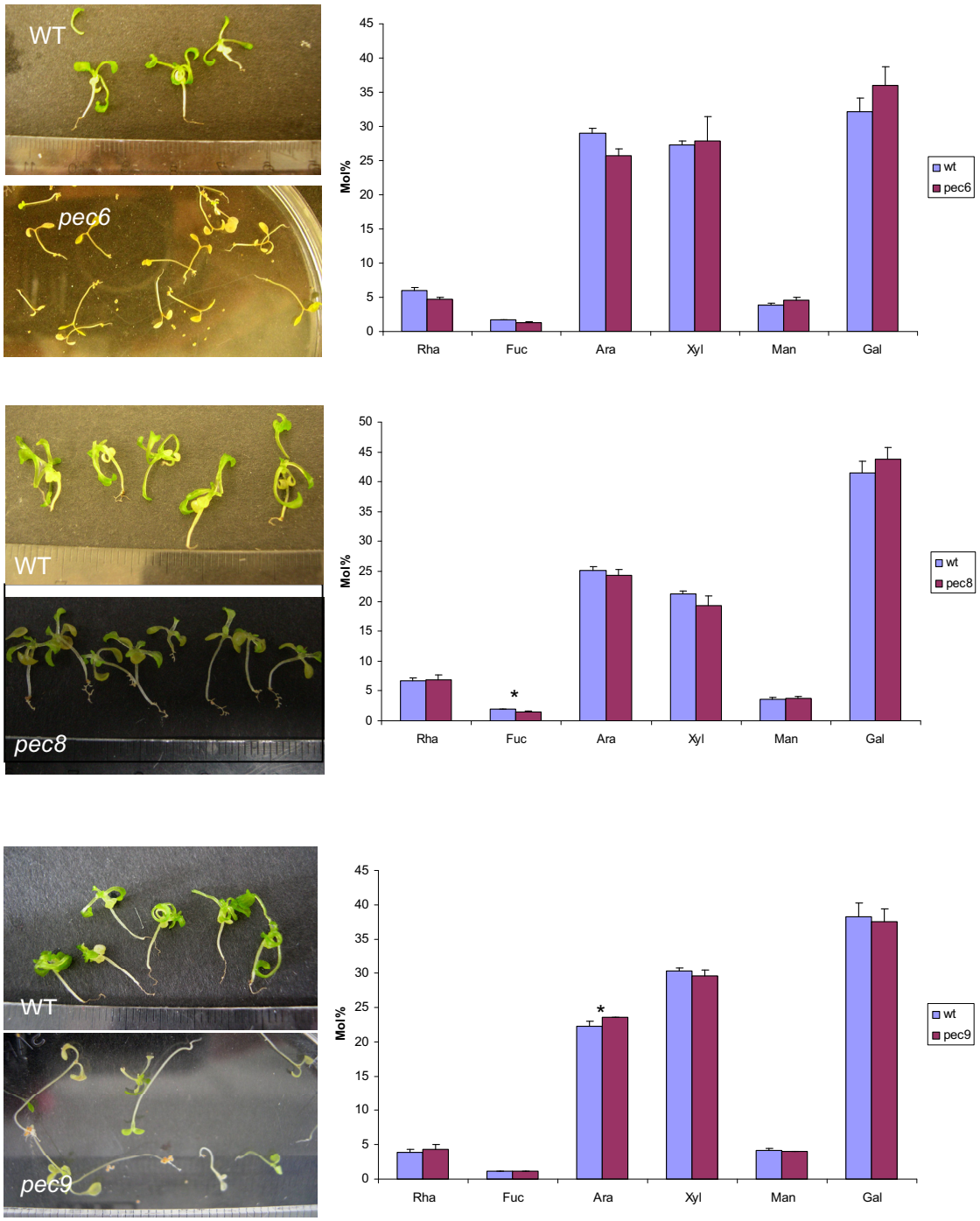
The second allele (*pec132-2*, Salk_ 043851C) did not reproduce the initial phenotype.



The second allele (*pec133-2*, Salk_137420) was not found.

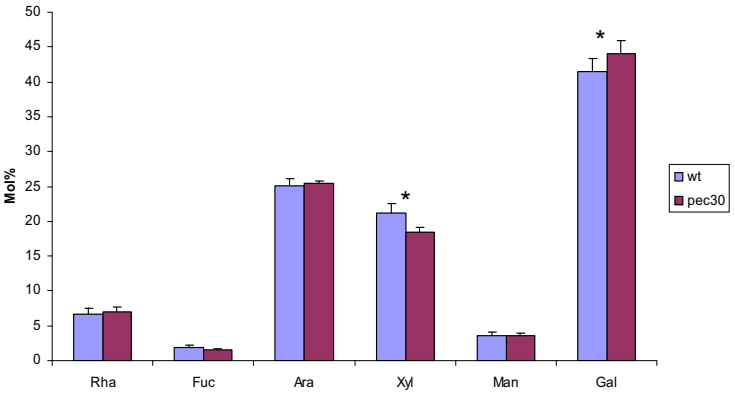
8.2 EMS mutants

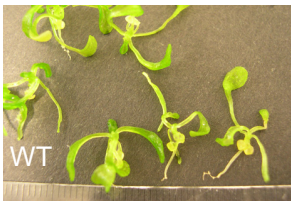
Figure 8.3 Liquid culture phenotype of WT and *pec* grown in presence of 0.25U/ml of endoPG/PME for 14 days and monosaccharide composition of cell wall material obtained from 4 day old etiolated seedlings. Asterisks indicate significant difference ($p<0.05$).

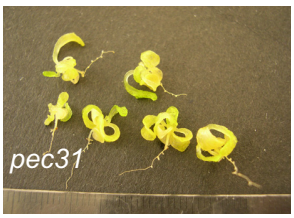


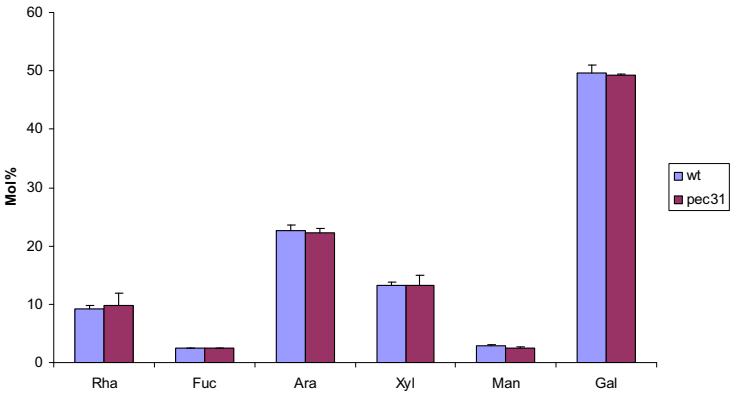

WT


pec30


Mol%
50
45
40
35
30
25
20
15
10
5
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
*
wt
pec30

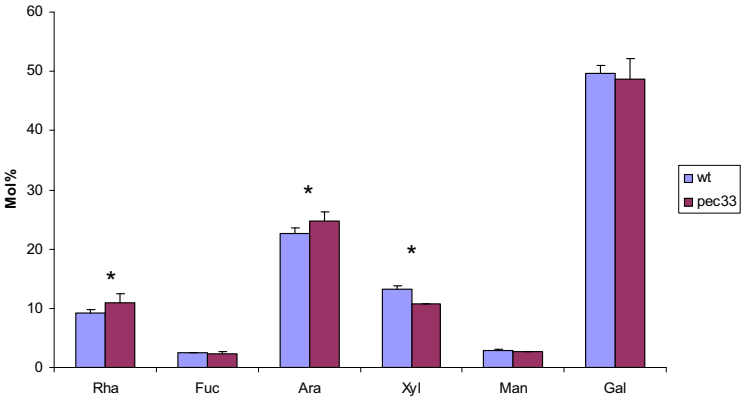

WT


pec31

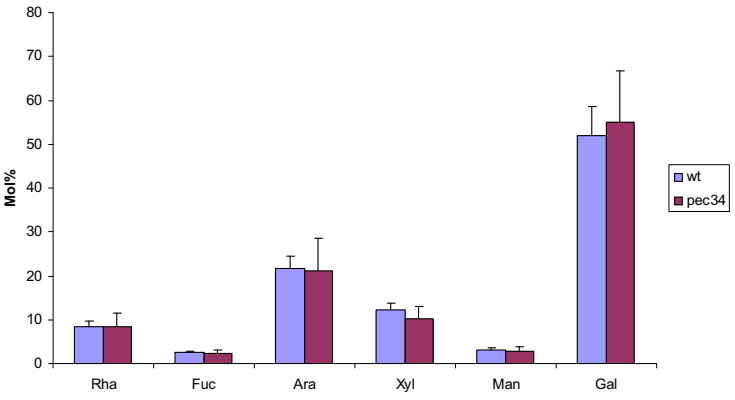

Mol%
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec31

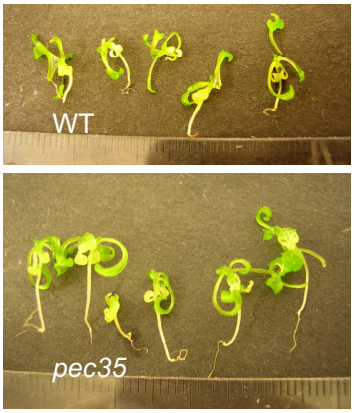

WT

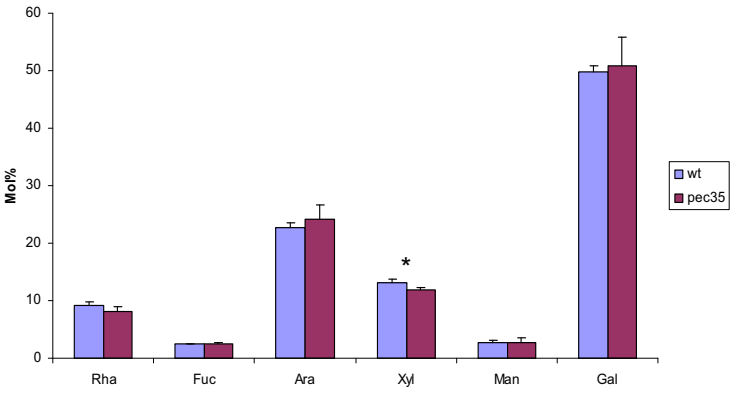

pec33

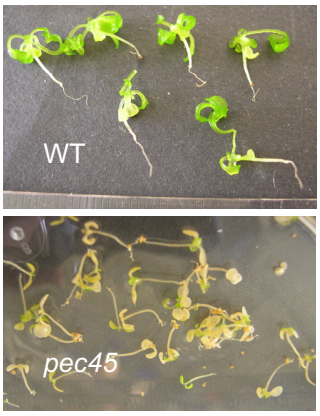

Mol%
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
*
*
wt
pec33

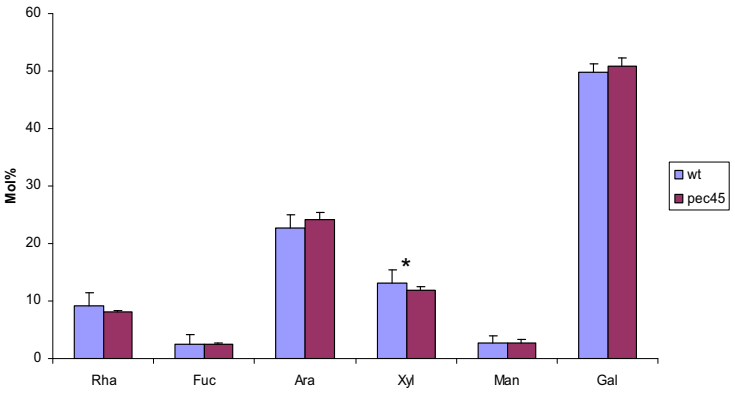

WT
pec34


Mol%
80
70
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec34


WT
pec35

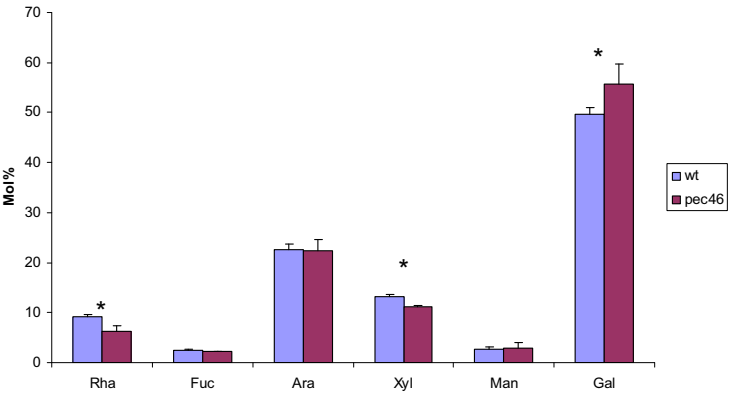

Mol%
60
50
40
30
20
10
0
*
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec35


WT
pec45


Mol%
60
50
40
30
20
10
0
*
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec45


WT


pec46


Mol%
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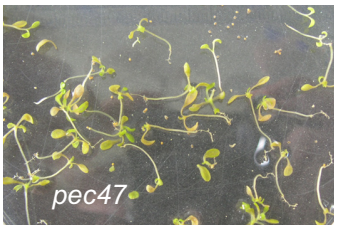
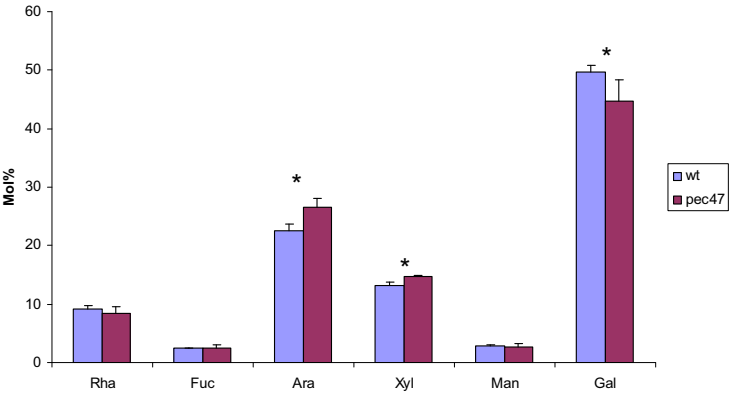
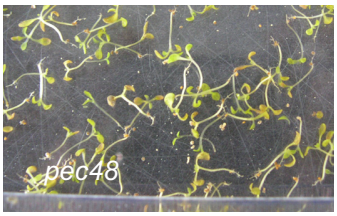
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
wt
pec46

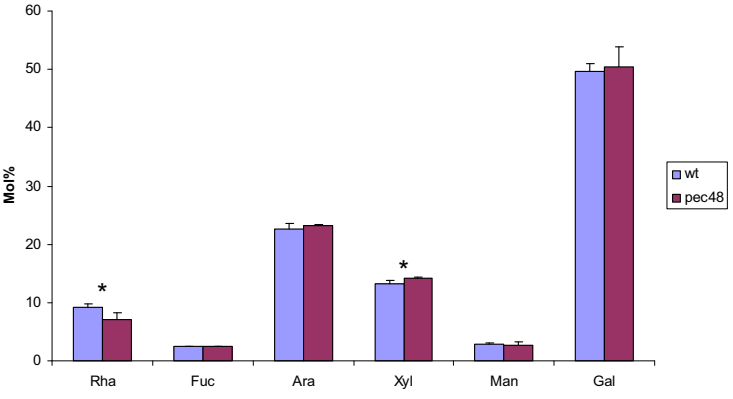

WT


pec47


Mol%
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
wt
pec47


WT


pec48


Mol%
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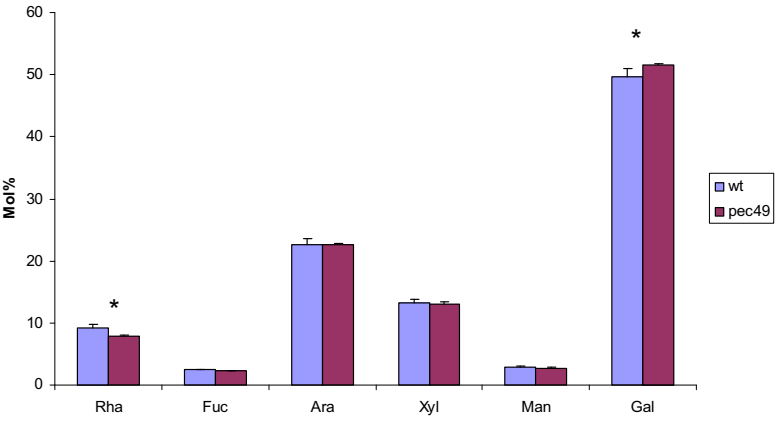
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
wt
pec48


WT


pec49


Mol%
60
50
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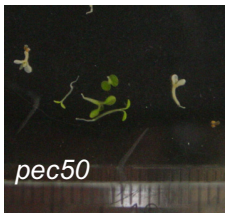
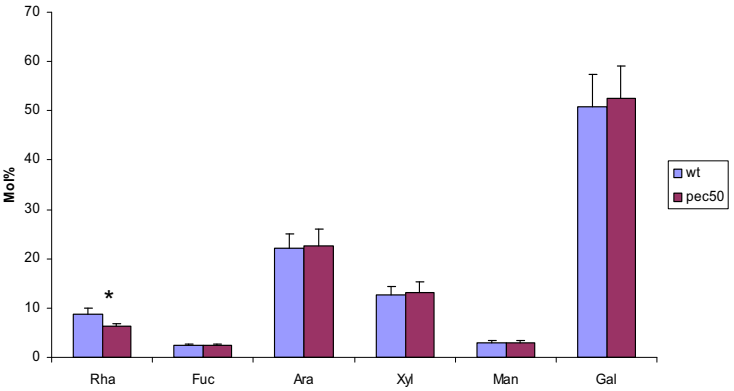
30
20
10
0
*
*
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec49


WT


pec50


Mol%
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60
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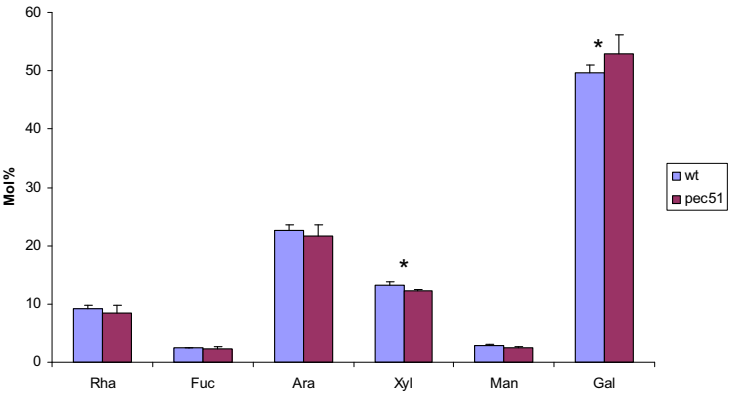
30
20
10
0
*
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec50


WT


pec51


Mol%
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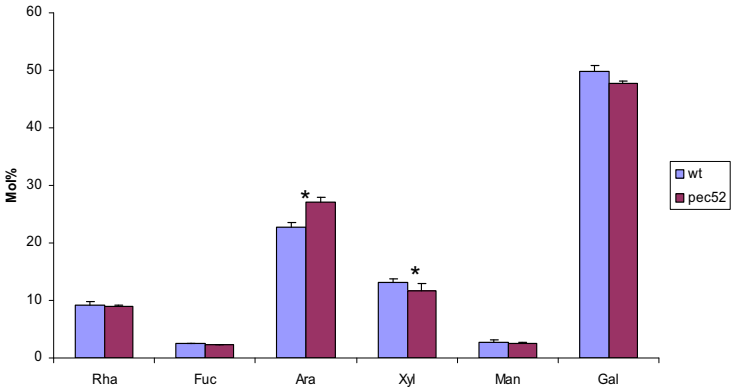
20
10
0
*
*
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec51


WT
pec52


Mol%
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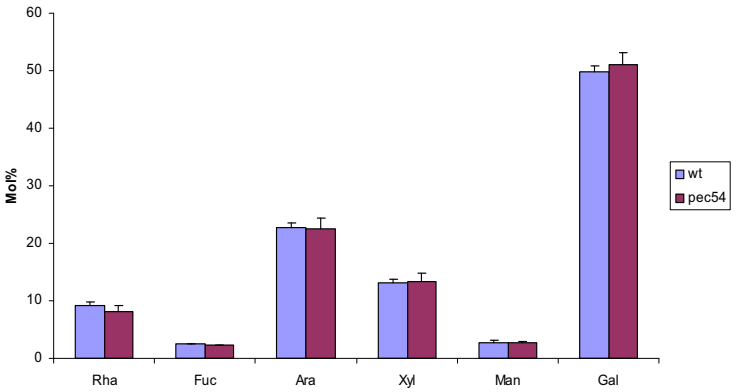
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
*
wt
pec52


WT
pec54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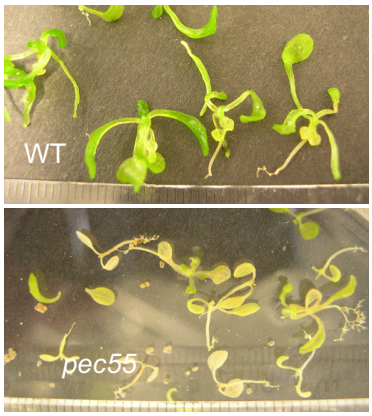
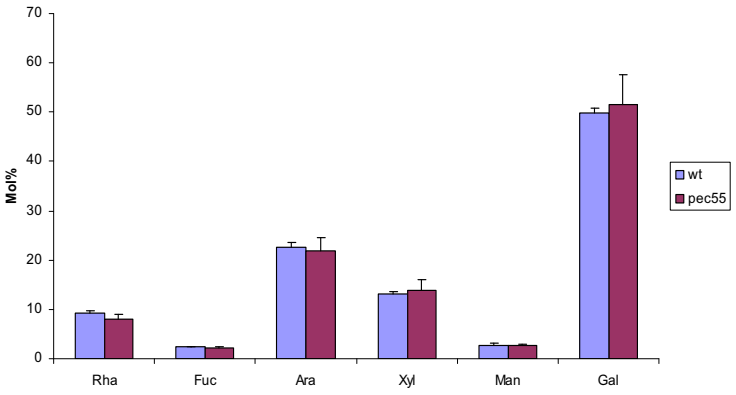
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec54


WT
pec55


Mol%
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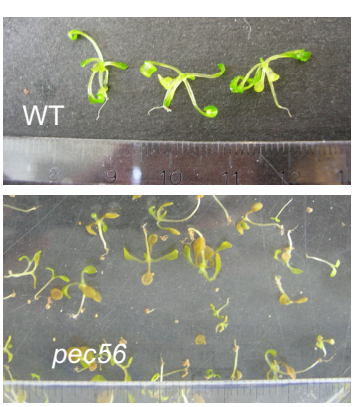
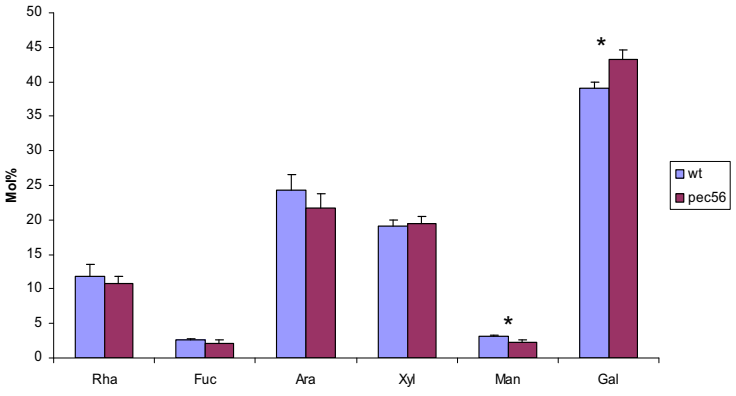
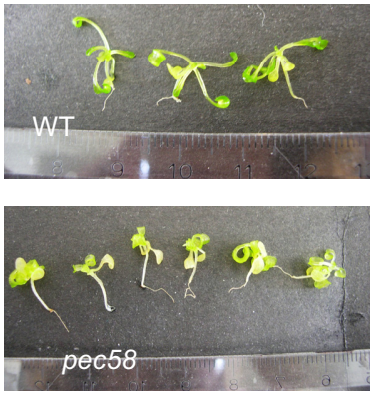
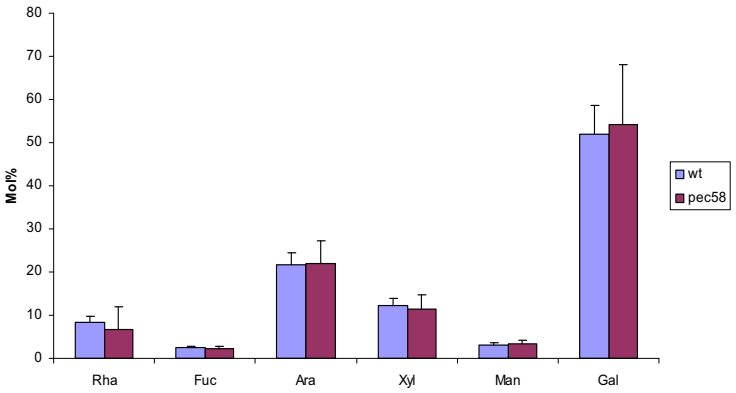
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec55


WT
pec56


Mol%
50
45
40
35
30
25
20
15
10
5
0
Rha
Fuc
Ara
Xyl
Man
Gal
*
*
wt
pec56


WT
pec58


Mol%
80
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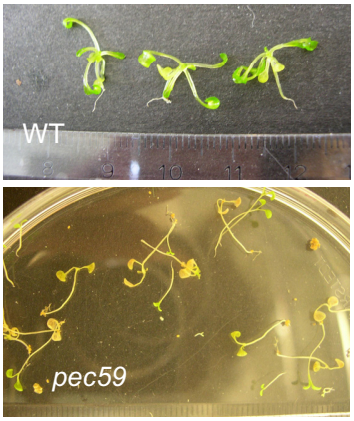
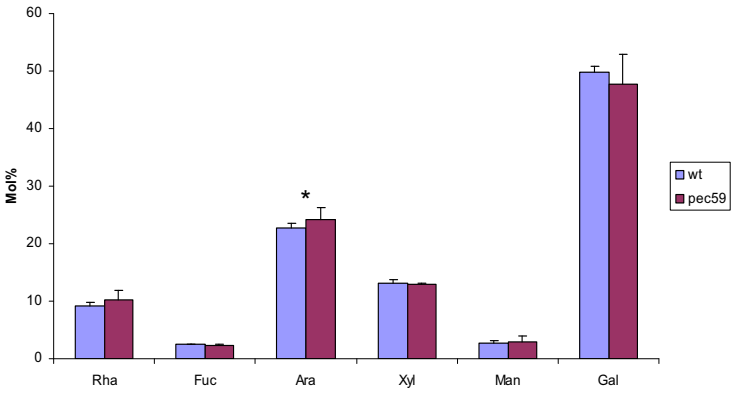
60
50
40
30
20
10
0
Rha
Fuc
Ara
Xyl
Man
Gal
wt
pec58


WT
pec59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Gal
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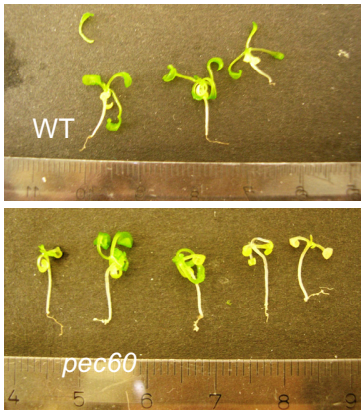
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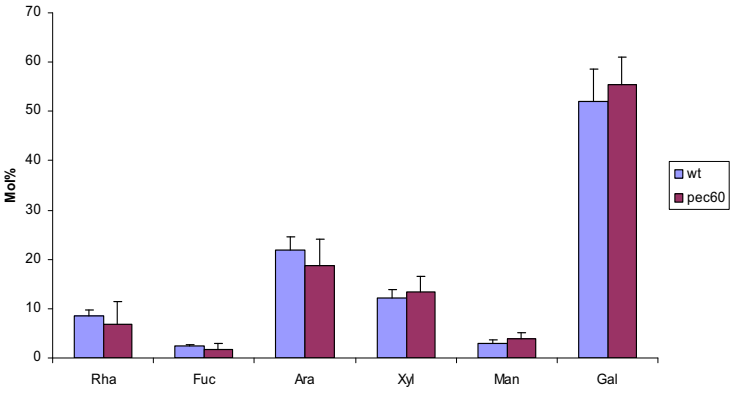
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WT
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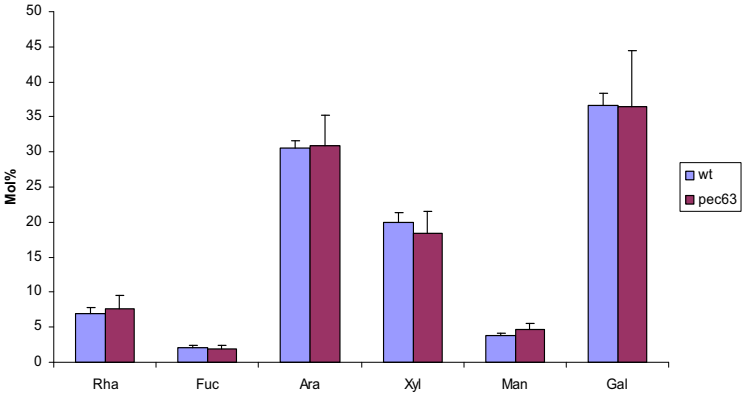
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Rha
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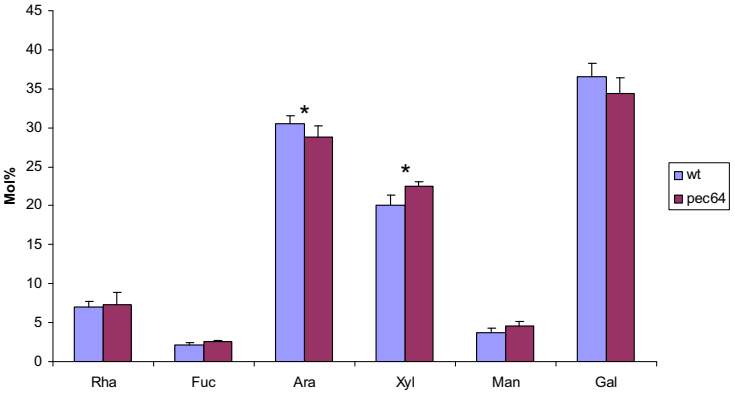
pec63


WT
pec64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pec64

8.3 QTL analysis

Table 8.3 List of 49 genes up-regulated in C24 compared to Col-0. Genes that are involved in cell wall structure and modification are highlighted in yellow. Data from the Weigel Lab (Lempe, J. et al. 2005).

C24 gcRMA	Col-0 gcRMA	c24/ col-0	Gene ID	gene name
10.24	1.84	5.57	At4g29200	beta-galactosidase (GH35)
9.49	2.91	3.26	At5g44440	FAD-binding domain-containing electron carrier
6.52	2.00	3.26	At2g33380	RD20 (responsive to desiccation 20); calcium binding
5.57	1.73	3.21	At5g67400	peroxidase 73 (PER73) (P73) (PRXR11)
8.06	2.67	3.02	At1g18710	AtMYB47 (myb domain protein 47); transcription factor
8.54	2.89	2.96	At1g61500	S-locus protein kinase, putative
5.93	2.07	2.87	At1g14100	FUT8; galactoside 2-alpha-L-fucosyltransferase
5.72	2.00	2.86	At5g43350	PHT1; carbohydrate:phosphate transporter
8.59	3.04	2.83	At5g15360	unknown protein
5.51	1.99	2.77	At1g05240	peroxidase, putative
5.34	2.02	2.65	At4g02270	pollen Ole e 1 allergen and extensin family protein
5.64	2.14	2.63	At4g26010	peroxidase, putative
5.47	2.09	2.61	At3g43790	ZIFL2; carbohydrate transporter
5.21	2.01	2.59	At3g62680	PRP3 (PROLINE-RICH PROTEIN 3); structural cell wall protein
6.74	2.62	2.57	At4g19030	aquaporin; NLM1 (NOD26-like intrinsic protein 1;1)
6.90	2.70	2.56	At1g66270	beta-glucosidase (PSR3.2)
5.70	2.28	2.51	At1g61800	GPT2 (glucose-6-phosphate/phosphate translocator 2); antiporter
7.54	3.08	2.44	At5g23020	methylthioalkylmalate synthase MAM3
5.15	2.14	2.41	At2g33790	pollen Ole e 1 allergen and extensin family protein
4.51	1.92	2.35	At5g17820	peroxidase 57 (PER57) (P57) (PRXR10)
5.72	2.44	2.34	At1g77330	1-aminocyclopropane-1-carboxylate oxidase / ACC oxidase
4.78	2.05	2.34	At4g25820	XTR9 (xyloglucan endotransglycosylase 9)
5.81	2.49	2.33	At4g33550	protease inhibitor/seed storage/lipid transfer protein (LTP) protein
5.10	2.19	2.33	At4g19690	IRT1 (IRON-REGULATED TRANSPORTER 1)
9.44	4.06	2.33	At4g23600	CORI3 (CORONATINE INDUCED 3); transaminase
4.80	2.07	2.32	At5g05500	pollen Ole e 1 allergen and extensin family protein
4.98	2.15	2.32	At4g34580	SRH1 (SHORT ROOT HAIR 1) transporter
4.54	1.99	2.29	At5g54370	late embryogenesis abundant (LEA) protein-related
5.92	2.60	2.28	At2g26560	PLP2 (PHOSPHOLIPASE A 2A); nutrient reservoir
5.63	2.48	2.27	At5g09530	hydroxyproline-rich glycoprotein family protein
5.31	2.34	2.27	At3g54590	ATHRGP1; structural constituent of cell wall
9.24	4.14	2.23	At3g61990	O-methyltransferase family 3 protein
4.06	1.84	2.21	At3g19430	late embryogenesis abundant (LEA) protein-related
4.40	2.05	2.15	At2g37870	protease inhibitor/seed storage/lipid transfer protein (LTP)
5.86	2.73	2.15	At3g53980	protease inhibitor/seed storage/lipid transfer protein (LTP)
4.44	2.08	2.14	At2g37130	peroxidase 21 (PER21) (P21) (PRXR5)
8.82	4.13	2.13	At1g50290	unknown protein
10.11	4.78	2.11	At4g15440	HPL1 (HYDROPEROXIDE LYASE 1); monooxygenase
11.47	5.43	2.11	At4g03060	AOP2 (ALKENYL HYDROXALKYL PRODUCING 2) oxidoreductase
6.92	3.28	2.11	At2g24980	proline-rich extensin-like family protein
7.15	3.40	2.10	At4g02330	ATPMEPCRB; pectinesterase
4.69	2.23	2.10	At4g38080	hydroxyproline-rich glycoprotein family protein
5.57	2.65	2.10	At5g59320	LTP3 (LIPID TRANSFER PROTEIN 3); lipid binding
4.48	2.14	2.09	At5g57530	xyloglucan:xyloglucosyl transferase
5.97	2.88	2.08	At5g34920	transposable element gene
4.35	2.10	2.07	At5g17330	GAD (Glutamate decarboxylase 1); calmodulin binding
5.00	2.46	2.03	At1g34510	peroxidase, putative
3.83	1.88	2.03	At4g28390	AAC3 (ADP/ATP CARRIER 3); ATP:ADP antiporter/ binding
7.46	3.68	2.03	At1g73260	trypsin and protease inhibitor family protein / Kunitz family protein
3.65	1.81	2.02	At5g12940	leucine-rich repeat family protein
5.20	2.58	2.02	At5g06640	proline-rich extensin-like family protein
7.56	3.75	2.02	At4g17470	palmitoyl protein thioesterase family protein
6.02	2.99	2.01	At1g76530	auxin efflux carrier family protein

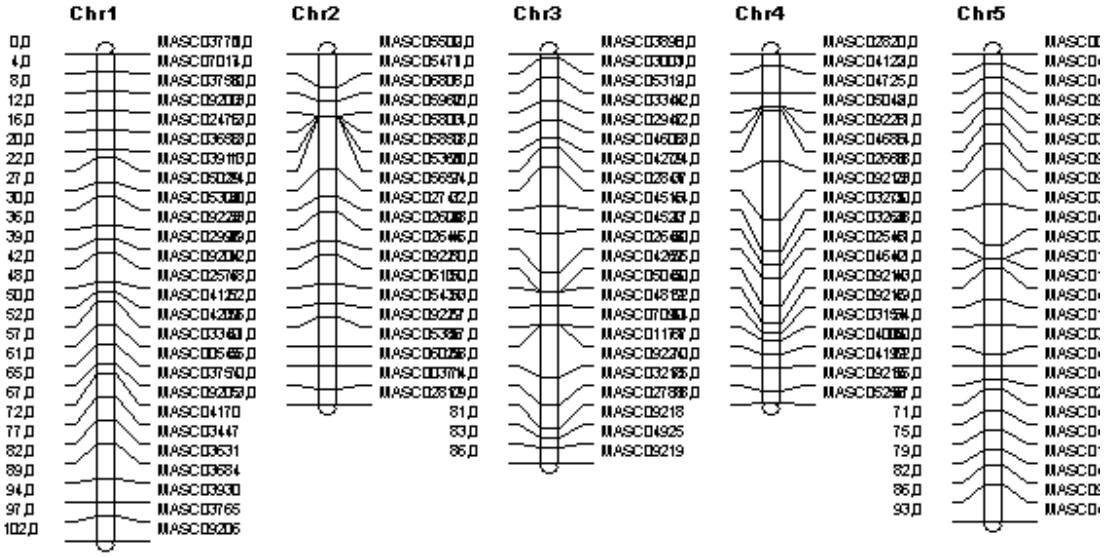

Fig.8.4 .Genetic map depicting the distribution of 110 genetic markers on the chromosomes of *A. thaliana* used in the genetic analysis of Col-0xC24 RILs (Torjek, O. et al. 2006).

8.4 Reverse genetic screen

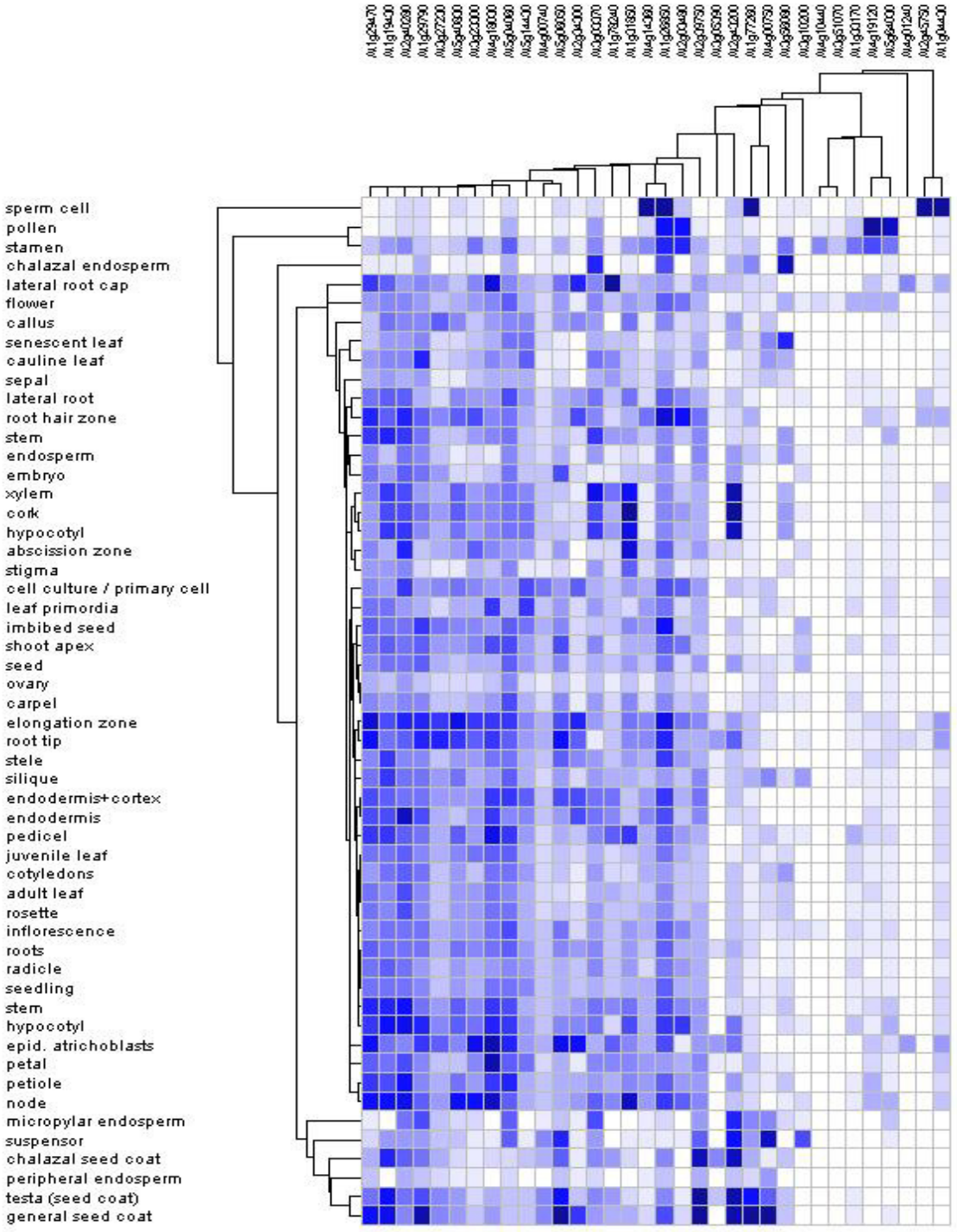

Figure 8.5 Genevestigator data of *A. thaliana* PMT organ specific expression. Organs for which data are included are indicated on the left side. The dendrograms indicate correlation of the gene expression pattern.

Table 8.4 Characterization of the PMT gene family in *A. thaliana*. Calculated protein properties, predicted localization by ARAMEMNON (Schwacke, R. et al. 2003) and experimental localization by LOPIT (Dunkley, T. P. et al. 2006) are presented for all the 35 members of the PMT protein family.

	Protein properties			ARAMEMNON		
Gene ID	Length(aa)	Mw (kDa)	pI	Consensus prediction	Score	LOPIT localization
AT1G04430	623	70.5	6.6	secretory pathway	15.7	Golgi
AT1G13860	603	68.0	6.8	secretory pathway	19.2	
AT1G19430	724	82.7	6.1	secretory pathway	10.1	Golgi
AT1G26850	616	69.6	7.0	secretory pathway	17.1	Golgi
AT1G29470	770	87.2	4.9	secretory pathway	13.4	Golgi
At1g29790	378	42.0	9.8	secretory pathway	28.8	Golgi
AT1G31850	603	68.8	8.9	secretory pathway	8.4	Golgi
AT1G33170	639	73.2	6.7	secretory pathway	8.2	
AT1G77260	655	74.4	6.7	secretory pathway	11.6	
AT1G78240	684	77.9	7.3	secretory pathway	1.7	Golgi
AT2G03480	606	67.8	7.4	secretory pathway	8.3	
AT2G34300	770	86.9	5.1	secretory pathway	20.7	Golgi
AT2G39750	694	78.4	7.9	secretory pathway	12.5	
AT2G40280	589	66.8	8.5	secretory pathway	28.4	Golgi
AT2G43200	611	70.2	9.6	secretory pathway	12.5	
AT2G45750	631	72.1	6.1	secretory pathway	15.1	
AT3G03370	145	16.9	9.6	secretory pathway	3	
AT3G05390	463	54.3	8.7	secretory pathway	14.1	
AT3G10200	591	67.6	7.3	secretory pathway	11.8	
AT3G23300	611	69.4	6.7	secretory pathway	20.8	Golgi
AT3G27230	410	45.9	9.7	secretory pathway	8.1	Golgi
AT3G51070	895	101.4	5.2	secretory pathway	12.8	
AT3G56080	357	41.1	8.2	unpredicted	0	
AT4G00740	600	67.6	9.1	secretory pathway	8.2	Golgi
AT4G00750	633	72.8	8.5	secretory pathway	13.6	Golgi
AT4G01240	659	75.2	5.4	secretory pathway	2.2	
AT4G10440	633	72.4	7.3	secretory pathway	7.4	
AT4G14360	608	69.2	6.5	secretory pathway	17.7	Golgi
AT4G18030	621	70.3	7.7	secretory pathway	12.4	Golgi
AT4G19120	600	68.3	7.5	secretory pathway	7.9	
AT5G04060	600	68.4	6.9	secretory pathway	15.5	Golgi
AT5G06050	682	77.7	7.3	secretory pathway	15.9	
AT5G14430	612	69.9	9.1	secretory pathway	13.1	Golgi
AT5G40830	414	46.5	8.3	secretory pathway	6.8	
AT5G64030	829	93.0	4.8	secretory pathway	6.6	Golgi

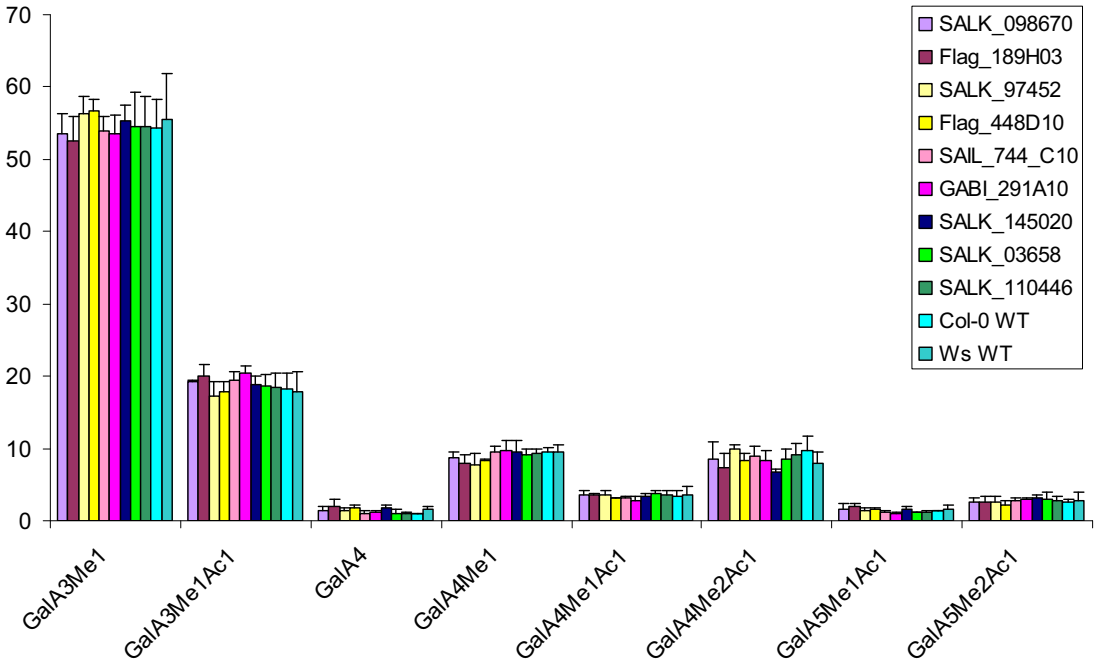

Figure 8.6. OLIMP of endoPG/PME digested cell wall material from T-DNA lines for 5 genes of the PMT gene family. Each class represents different oligogalacturonides (OGAs). GalA – galacturonic acid Me – methyl ester, Ac acetyl ester, numbers indicate GalA monomers and esters. Error bars represent standard deviation, N=4.

9 Eidesstattliche Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt habe, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat. Die Bestimmungen dieser Promotionsordnung sind mir bekannt.

Nino Nikolovski

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Some of the data gathered in my research were presented to audiences at MPI-MP and Potsdam University, the Plant Research Laboratory at MSU, ASPB and Gordon research conferences and the International Cell Wall Meeting. I am grateful for the invitation to speak and present my data, and for the comments and suggestions that I received on those occasions. I am also very grateful to my friends for reading through the entire dissertation and providing valuable critique and comments on matter of both substance and stile.

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NN