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Secretory pathway in plant cell wall polysaccharide biosynthesis and FT-IR spectroscopy methods in plant cell wall composition analysis

Úloha sekreční dráhy v biosyntéze polysacharidů buněčné stěny rostlin a FT-IR spektroskopické metody v analýze složení buněčné stěny

Bachelor's thesis

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Statement:

I hereby state that I have completed this thesis by myself and that I have properly cited all literature and other information sources I have used. Neither this thesis nor its parts have been submitted to achieve any other academic title(s).

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Contents

Abstract

All plant cells are encapsulated in a cell wall that determines the cells' shape and size and is essential to many of their vital processes. The cell wall of streptophyte plants is composed mainly of polysaccharides of high molecular weight. Cellulose, the main constituent of the plant cell wall, is synthesized by protein complexes bound to the PM, while hemicelluloses and pectins are synthesized in the Golgi apparatus. Several proteins that participate in the synthesis of cell wall polysaccharides have been identified, but hundreds of them remain to be discovered. Secretory pathway plays an important role in plant cell wall biogenesis as it transports cellulose synthase complexes and noncellulosic polysaccharide molecules to the cell surface. Some regulatory mechanisms that might be involved in cell wall material secretion, such as actin cytoskeleton, Ca^{2+} gradient or PIP kinases have been proposed; however, the regulation of this process is very complex and far from being understood. FT-IR spectroscopy is a method that can detect molecular vibrations and provide information about chemical composition of virtually all substances. It has been used successfully in screens for cell wall mutants, the study of interactions between cell wall polymers, as well as other areas of cell wall biology. FT-IR spectroscopy is proposed as a suitable tool for future research of the role of the secretory pathway in plant cell wall biogenesis.

Keywords

Cell wall, secretory pathway, FT-IR spectroscopy, cellulose, pectin, hemicellulose

Abstrakt

Všechny rostlinné buňky jsou obklopeny buněčnou stěnou, která ustavuje jejich velikost a tvar a je nezbytná pro mnoho zásadních buněčných procesů. Buněčná stěna streptofytních rostlin je tvořena převážně vysokomolekulárními polysacharidy. Celulóza, hlavní složka buněčné stěny rostlin, je syntetizována transmembránovými proteinovými komplexy přímo na povrchu buňky, zatímco hemicelulózy a pektiny jsou tvořeny v Golgiho aparátu. Bylo popsáno několik proteinů účastnících se syntézy těchto polysacharidů, nicméně stovky dalších teprve čekají na objevení. Proteiny nutné pro syntézu celulózy a molekuly hemicelulóz a pektinů syntetizované v Golgiho aparátu jsou transportovány na povrch buňky sekreční drahou, tato dráha tedy hraje důležitou roli v biogenezi buněčné stěny. Hypotézy o regulaci sekrece komponent buněčné stěny počítají s působením aktinového a mikrotubulárního cytoskeletu, vápníkového gradientu nebo PIP kináz; komplexní regulace tohoto procesu však zdaleka není popsána. FT-IR spektroskopie je metoda schopná detekce molekulárních vibrací, s jejíž pomocí je možné získat informace o chemickém složení prakticky všech látek. Tato metoda je úspěšně používána v identifikaci mutantů s abnormální buněčnou stěnou, ve studiu interakcí mezi jednotlivými složkami buněčné stěny a v dalších experimentech týkajících se buněčné stěny. FT-IR spektroskopie je navržena jako ideální nástroj pro budoucí výzkum role sekreční dráhy v biogenezi buněčné stěny rostlin.

Klíčová slova

Buněčná stěna, sekreční dráha, FT-IR spektroskopie, celulóza, pektiny, hemicelulózy

List of abbreviations

Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
ATR	attenuated total reflectance
CESA	cellulose synthase
CSL	cellulose synthase-like
EGFP	enhanced green fluorescent protein
flax	*Linum usitatissimum* L. cv. Novotorzhskii
FT	Fourier transform
FT-IR	Fourier transform infrared
GA	Golgi apparatus
GalAT	galacturonosyltransferase
GATL	galacturonosyltransferase-like
GAUT	galacturonosyltransferase
GAX	glucuronoarabinoxylan
GFP	green fluorescent protein
GM	glucomannan
HG	homogalacturonan
IR	infrared
kam1	katamari1
KOR1	korrigan1
LatB	latrunculin B
LDA	linear discriminate analysis
LOF	loss of function
maize	*Zea mays* L.
MIR	mid-infrared
MLG	mixed-linkage glucan
MTs	microtubules
NIR	near-infrared
PC	principal component score
PCA	principal component analysis
pea	*Pisum sativum* L.
PIP5K	phosphatidylinositol-4-phosphate 5-kinase
PM	plasma membrane
RG-1	rhamnogalacturonan 1
RG-2	rhamnogalacturonan 2
RGXT	rhamnogalacturonan xylosyltransferase
rice	*Oryza sativa* L.
rose	*Rosa sp.* L.
spruce	*Picea abies* (L.) Karst.
TGN	Trans Golgi Network
tobacco	*Nicotiana sp.*L.
VHA-a1	vacuolar V-ATPase subunit a1
XG	xyloglucan
XGA	xylogalacturonan
XGD	xylogalacturonan deficient
XY	xylan
YFP	yellow fluorescent protein
ZPD	zero path difference

1. Introduction

The plant cell wall is a more or less rigid structure that encapsulates all plant cells and determines their shape and size. It plays crucial roles in processes such as growth and development, defense against pathogens or cell to cell signaling and transport. Plant cell walls are also of utmost importance to mankind, as we use them to produce food, clothing, fuels and paper (Geisler et al., 2008; Alonso-Simón et al., 2011). The knowledge and understanding of plant cell wall biogenesis and functions are thus essential for progress in both basic and applied research. Although our knowledge of plant cell wall chemical composition is relatively good, most mechanisms responsible for its biogenesis remain yet to be discovered.

Fourier Transform Infrared (FT-IR) spectroscopy is a classical method used in the analysis of small molecules as well as large biopolymers. A variety of information about the studied substance can be then obtained from its IR spectrum, including chemical structure, redox state, bond properties, hydrogen bond presence and more (Barth, 2007).

The first part of this overview summarizes current knowledge about plant cell wall biogenesis with focus on the role of the secretory pathway in this process; the second part describes basic principles of FT-IR spectroscopy. Several applications of this method in the study of the cell wall are presented in the end with the intention to show that FT-IR spectroscopy is a powerful tool that could provide more insights into mechanisms of cell wall biogenesis in the future.

2. Secretory pathway in plant cell wall polysaccharide biosynthesis

2.1 Cell wall composition

The plant cell wall is a network structure formed mainly by polysaccharides of high molecular weight, which are embedded in an aqueous solution, with important components of proteins, glycoproteins and in many cases also other substances, such as lignin (Liepman et al., 2005; Geisler et al., 2008; Jensen et al., 2008). As this work is focused on cell wall polysaccharides, other cell wall components won't be discussed further on. The main classes of cell wall polysaccharides are cellulose, hemicelluloses and pectins (Somerville et al., 2004).

Cellulose is the basic polysaccharide that constitutes the cell wall of streptophyte plants and the most abundant biopolymer on the planet (Kimura et al., 1999). A cellulose molecule is formed by a chain of β-1,4-linked glucose residues. In cell walls, cellulose is present in the form of para-crystalline microfibrils, which are composed of 30-36 glucan chains connected by hydrogen bonds (Kimura et al., 1999; Paredez et al., 2006). Hemicelluloses, which are branched polysaccharides formed by a heavily substituted neutral sugar backbone, crosslink cellulose microfibrils by hydrogen-bonding to their surface. There are four basic kinds of hemicelluloses: xyloglucans (XG), glucuronoarabinoxylans (GAX – esp. in type II grass cell walls), glucomannans (GM) and mixed-linkage glucans (MLG) (Somerville et al., 2004; Lerouxel et al., 2006). Pectins are defined as polysaccharides with a high content of galacturonic acid residues. The main classes of pectins are homogalacturonan (HG), rhamnogalacturonan 1 (RG-1), rhamnogalacturonan 2 (RG-2) and xylogalacturonan (XGA) (Sterling et al., 2006; Jensen et al., 2008). Some highly specialized cell walls, such as in the case of plasmodesmata (there directly involved in size exclusion limit regulation), pollen tubes or newly synthesized walls during cytokinesis or after a wound, also contain callose, which is an amorphous β-1,3-glucan chain (Verma and Hong, 2001).

The relative amounts of polysaccharide components listed above depend on the type of the cell wall, its developmental stage, and they also vary among different plant taxa. The primary cell wall usually contains similar amounts of cellulose, hemicelluloses and pectins;

the secondary wall has usually higher amounts of cellulose and less of the other polysaccharides (Jensen et al., 2008; Sandhu et al., 2009). The content of pectin goes up to 35% in the primary walls of dicotyledons and nongraminaceous monocotyledons, while graminaceous monocots, i.e. cereals and grasses, usually only have about 10% pectin in their primary walls (Sterling et al., 2006). While the most abundant hemicellulose in dicotyledons and non-graminaceous monocotyledons is XG, grasses contain larger amounts of xylan (Kerr and Fry, 2003). A model of an *Arabidopsis* leaf mesophyll cell wall is presented in figure 1.

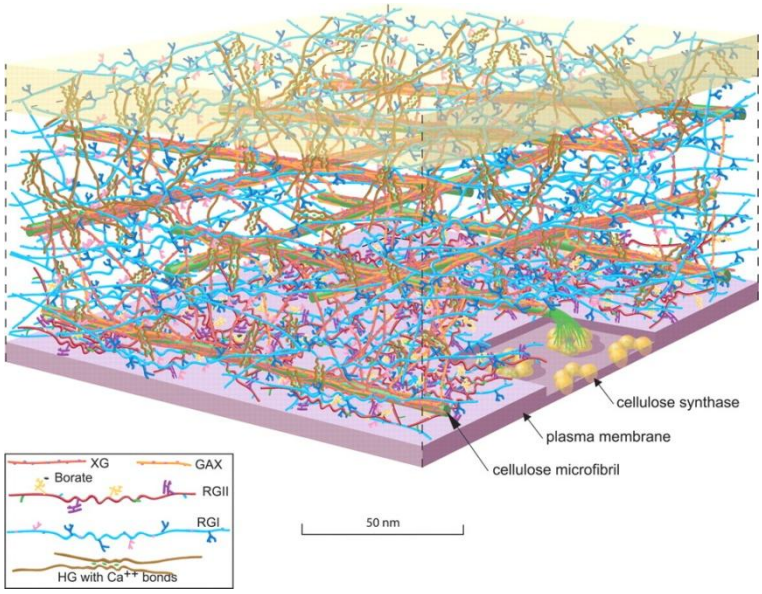


Figure 1 *Model of the cell wall of an Arabidopsis leaf mesophyll cell. The amount of cellulose is reduced for better clarity; the amount of other polymers is reduced as well in approximately the same ratio. Due to this reduction, the hemicellulose cross-links (XG and GAX) are much longer in the figure than in reality. Courtesy of (Somerville et al., 2004)*

2.2 Cell wall polysaccharide biosynthesis

2.2.1 Model systems used in the study of plant cell wall biogenesis

Our knowledge of the mechanisms responsible for the synthesis of the plant cell wall is still very limited. Attempts for biochemical purification of enzymes responsible for cell wall polysaccharide biosynthesis, as well as mutant screens and reverse genetic methods, have so far only been successful in several cases (Jensen et al., 2008). The proteins involved in the synthesis of the cell wall polysaccharides are present in very low concentrations in the cell, they are membrane bound and not very stable, which makes them difficult to isolate and study biochemically; also, wide genetic redundancy among these genes is probably a great complication in approaches based on mutational genetics (Jensen et al., 2008; Sandhu et al., 2009)

Heterologous expression systems have been used with relative success to identify genes and proteins involved in the cell wall synthesis. *GAUT1*, a galacturonosyltransferase required for the synthesis of pectin in *Arabidopsis*, has been characterized when transiently expressed in the human kidney HEK293 cell line. (Sterling et al., 2006); AtCslA9, a β-glucomannansynthase that catalyzes the polymerization of hemicellulosic backbones, has been expressed and characterized in a similar manner in *Drosophila* Schneider 2 cells (Liepman et al., 2005).

Germinating pollen grains and developing root hairs are highly specialized plant cells with very fast apical growth; both of these systems are widely used to study the cell wall biogenesis. New cell wall material must be constantly transported to the growing tip and the cell wall must be continuously rebuilt for such growth to be possible (Chen et al., 2007; Ischebeck et al., 2008; Fan et al., 2011). This makes the growing pollen tube and root hair perfect systems for the study of mechanisms by which cell wall material, synthesized within the cell, is transported to the cell surface and incorporated into the wall.

Another question that has to be addressed if we want to understand how plants make their cell walls is what happens with the polysaccharide building blocks once they're synthesized and transported to the cell surface. Experiments conducted with cell suspension cultures of maize and rose fed with radioactive-labeled monosaccharides have provided some insight into such *in muro* polysaccharide modifications (Thompson and Fry, 1997; Kerr and Fry, 2003).

The Paul Knox lab has developed a wide range of monoclonal antibodies against specific cell wall components over the past two decades, including JIM5 against de-esterified pectins, JIM7 against esterified pectins, and many more (Knox et al., 1991; Clausen et al., 2003). These commercially available antibodies are widely used in cell wall research (Chen et al., 2007; Jensen et al., 2008). FM4-64 is an amphiphilic fluorescent dye that can only enter living cells by endocytosis and marks the PM and endomembranes including endocytic and secretory vesicles (Parton et al., 2001). This dye has been used extensively in the research of plant vesicle trafficking and its role in cell wall biogenesis (Dettmer et al., 2006; Fan et al., 2011).

2.2.2 Synthesis of cellulose and callose

Cellulose and callose are, unlike other cell wall polysaccharides, synthesized by protein complexes bound to the PM and deposited directly into the wall.

The transmembrane cellulose synthase complex of higher plants, which is one of the largest protein complexes known, is often called a “rosette”. It is believed to be a hexameric complex formed by six subunits, each of which contains six cellulose synthase *(CESA)* proteins along with multiple other proteins (Kimura et al., 1999). Each of the *CESA* subunits synthesizes an individual β-1,4-glucan chain from cytosolic UDP-glucose and the complex leaves a complete new cellulose microfibril in the cell wall (Somerville et al., 2004; Lerouxel et al., 2006). The elongation of a cellulose microfibril forces the rosette to move through the PM, usually in a direction perpendicular to the direction of cell elongation. There is good evidence that this pattern is maintained by cortical MTs, which serve as guiding tracks of the active rosette complexes (Paredez et al., 2006).

Plant genomes encode multiple CESA genes; the model plant *Arabidopsis thaliana* features 10 CESA homologues. It has been proven that at least three different CESA protein isoforms are required to form a functional cellulose synthase complex, indicating that multiple CESA genes are not redundant, but their functions are slightly different. For example, it has been hypothesized that cellulose synthesis in primary and secondary cell walls is carried out by different CESA homologues (Richmond and Somerville, 2000; Taylor et al., 2003; Lerouxel et al., 2006).

Although cellulose microfibrils are deposited directly into the cell wall upon synthesis at the PM, the secretory pathway is essential for cellulose biosynthesis. KORRIGAN1 (KOR1) is a β-1,4-endoglucanase necessary for cellulose synthesis, although its precise biochemical function remains unclear. Experiments with GFP-KOR1 fusion protein revealed that KOR1 localizes to the PM, GA and early endosomes and that its regulated secretory pathway dependent cycling between these compartments is required for cellulose biosynthesis (Robert et al., 2005). *Arabidopsis* CESA6 has also been shown to be present at the PM, GA and in secretory vesicles (Paredez et al., 2006). These results strongly support the hypothesis that cellulose deposition is regulated by the cycling of proteins involved in microfibril synthesis between the PM and intracellular pools (Lerouxel et al., 2006).

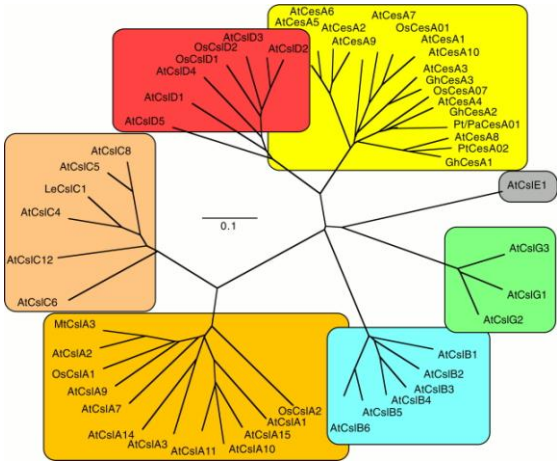


Figure 2 *An unrooted phylogenetic tree of the CESA superfamily. The tree was created by the software ClustalX 1.8 from publicly available protein sequences, color boxes represent subfamilies. At - Arabidopsis; Gh - cotton; Le - tomato; Mt - Medicago truncatula; Os - rice; Pt - Populus tremuloides; Pt/Pa - Populus tremula × Populus alba. Courtesy of (Richmond and Somerville, 2000)*

The synthesis of callose is considered to follow similar patterns as the synthesis of cellulose. Sequence homologies between CESA genes and putative callose synthases, including identical transmembrane domains, support this hypothesis. However, we have considerably less information about the biosynthesis of callose compared to cellulose, partly because of problems with purification of enzymes that catalyze callose synthesis (Verma and Hong, 2001).

2.2.3 Synthesis of hemicelluloses

Hemicelluloses, i.e. XG, GAX, GM and MLG, are, unlike cellulose, synthesized from monosaccharide precursors in the GA and transported to the cell wall in secretory vesicles (Northcote and Pickett-Heaps, 1966; Kerr and Fry, 2003; Liepman et al., 2005). Once these polysaccharides are secreted to the PM surface, they are further processed and incorporated into the wall network (Thompson and Fry, 1997; Kerr and Fry, 2003).

In silico analyses of *Arabidopsis* and other plant species' genomes have revealed many cellulose synthase-like (CSL) genes based on their sequence similarity to the *CESA* genes. The *CSL* genes, which can be further divided into different families, are part of the Cellulose synthase superfamily along with the *CESA* genes (fig. 2). All *CSL* genes had originally been predicted to encode membrane-bound glycosyltransferases that locate to the GA and are involved in the synthesis of non-cellulosic cell wall polysaccharides (Richmond and Somerville, 2000). However, recent results indicate that some of the *CSL* genes' products have β-1,4-glucan synthase activity at the PM in the tip regions of growing root hairs (Park et al., 2011), suggesting that the *CSL* family members may also carry out cellulose synthase functions in the synthesis of the cell wall.

To test the *CSL* genes' glycosyltransferase activity, Liepman et al. transfected *Drosophila* S2 cells with several *CSL* cDNA clones from *Arabidopsis* and rice and conducted enzyme activity assays with the recombinant proteins (Liepman et al., 2005). The microsomal fractions from lysates of transformed S2 cells were isolated, as all the *CSL* proteins were expected to be membrane bound. These fractions were incubated with a mixture of radiolabeled nucleotide diphosphate-sugars. GFP-transformed cells were used as negative controls and maize and pea microsomal fractions as positive controls. In this assay, the cells expressing the AtCslA9 protein incorporated GDP-mannose and GDP-glucose into insoluble polymers. Further experiments led to the conclusion that AtCslA9 produces β-mannan if it has access to GDP-mannose, β-glucan if it has access to GDP-glucose and β-linked mixed polymer when it has access to both nucleotide-sugars. The protein was thus characterized as β-glucomannan synthase (Liepman et al., 2005). These results strongly support the hypothesis that hemicelluloses are synthesized in the GA by glycosyltransferases encoded by genes of the *CSL* families.

There is good evidence that hemicelluloses are further processed upon their incorporation into the cell wall. Interestingly though, this processing is very different in dicots and graminaceous monocots, as shown by studies with rose and maize cell suspension cultures fed with radiolabeled arabinose (Thompson and Fry, 1997; Kerr and Fry, 2003). In the rose cell culture, radiolabeled arabinose was incorporated into XG molecules which were then deposited into the cell wall. The average molecular weight of individual XG molecules after 2 days was approximately 5 times lower than immediately after the labeling. At the same time, about 25% percent of the XG, which had originally been incorporated into the cell wall, disappeared into the culture medium. These results suggest that in dicots, the "loose end" parts of the XG molecules that don't form hydrogen bonds with cellulose microfibrils are cleaved and discarded from the cell wall (Thompson and Fry, 1997). The situation was quite different in the maize culture. Here, the radiolabeled arabinose was incorporated not only to XGs, but also to xylan molecules. The hemicellulose molecules found in the wall only 15 minutes after the labeling increased in size about 40-fold compared to the molecules present in the protoplasm. This indicates that after secretion into the cell wall, the xylan and XG molecules of grasses probably form large polymers, possibly by the mechanisms of transglucosylation. Some of the hemicellulose molecules were also trimmed later on as some radioactivity was found in the

culture medium, but this occurred at much lower levels than in the rose culture (Kerr and Fry, 2003).

2.2.4 Synthesis of pectins

Pectins, that is HG, RG-I, RG-II and XGA, are synthesized in the GA like hemicelluloses and then transported to the cell surface by the secretory pathway (Northcote and Pickett-Heaps, 1966; Jensen et al., 2008). The enzymes predicted to be necessary for pectin biosynthesis include glycosyltransferases, methyltransferases and acetyltransferases. The proteins that have been proven to play a role in pectin biosynthesis include a HG galacturonosyltransferase, RG-2 xylosyltransferase and XGA xylosyltransferase (Egelund et al., 2006; Sterling et al., 2006; Jensen et al., 2008). A vast number of other proteins need to be identified if we want to fully understand the mechanisms of pectin biosynthesis.

An enzyme with HG galacturonosyltransferase (HG GalAT) activity was partially purified from an *Arabidopsis* cell culture and its sequence analysis revealed that it was a product of the gene At3g61130. The protein, when transiently expressed in human kidney cell line HEK293, incorporated GalA from radiolabeled UDP-GalA into HG molecules and was thus named galacturonosyltransferase 1 (GAUT1). A GAUT1 antiserum was shown to block GalAT activity in *Arabidopsis* cell culture, confirming that GAUT1 was indeed a GalAT. Furthermore, a HG specific exopolygalacturonase cleaved 98.9% of the GAUT1 product, proving the enzyme was producing HG (Sterling et al., 2006). *In silico* analyses of the GAUT1 amino acid sequence revealed that the genome of *Arabidopsis* contained 14 coding regions with 56-84% sequence similarity to GAUT1 and 10 coding regions with 42-53% sequence similarity. These were named GAUT 2-15 and GATL (GalAT-like) 1-10, respectively. The authors suggest that all these genes form a GAUT1-related gene superfamily and encode putative GalATs involved at different stages of pectin biosynthesis (Sterling et al., 2006).

Two loci in the *Arabidopsis* genome, At4g01770 and At4g01750, were identified as putative cell wall polysaccharide glycosyltransferases by a bioinformatic approach and their functional characteristics were studied (Egelund et al., 2006). The proteins encoded by these genes were transiently expressed in insect cells, which were then incubated with different combinations of radiolabeled nucleotide diphosphate-sugars and monosaccharide acceptors. These assays showed that both gene products catalyzed the transfer of D-xylose

from UDP-xylose onto L-fucose by an α-(1,3) linkage, which makes them (1,3)-α-D-xylosyltransferases. Such a linkage is only known in the pectin RG-2; the two proteins were thus named Rhamnogalacturonan Xylosyltransferase 1 and 2 (RGXT1 and RGXT2), respectively (Egelund et al., 2006). The localization of RGXT1 to the GA was confirmed by colocalization of a RGXT1-EGFP construct with a BODIPY TR Ceramide dye, which is known to stain the GA (fig.3). RGXT-2 localized in the same manner (Egelund et al., 2006).

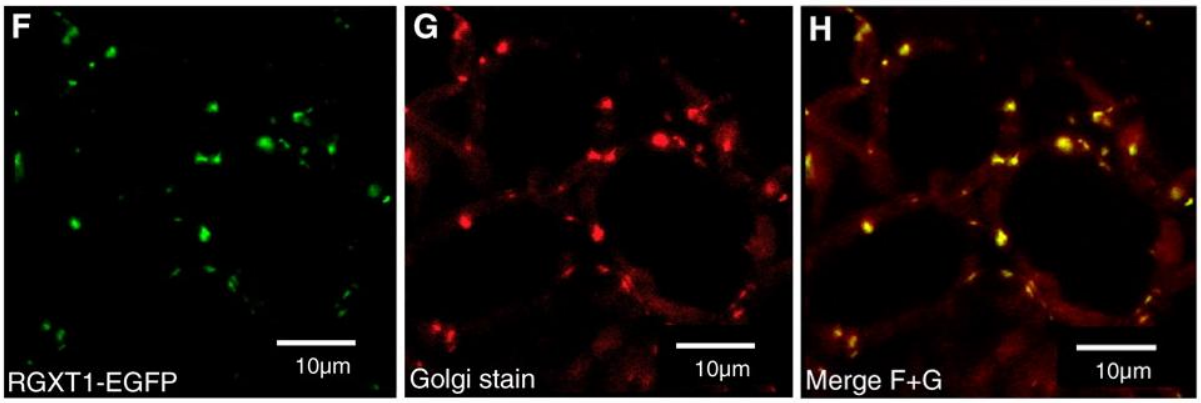


Figure 3 *Subcellular localization of RGXT1. GFP fluorescence in cells expressing RGXT-EGFP fusion protein (F); signal from the BODIPY TR dye which localizes specifically to the GA (G); images F and G merged together show clearly that RGXT-EGFP colocalizes with the BODIPY TR dye, proving that RGXT1 is a GA-resident protein. Courtesy of (Egelund et al., 2006)*

Another *Arabidopsis* genome locus At5g33290 was predicted to encode a type II membrane protein with glycosyltransferase activity localized in the secretory pathway. T-DNA insertion mutants in this locus contained approximately 25% less cell wall xylose compared to the wild type, the content of other cell wall monosaccharides wasn't affected by the mutation. Immunological assays with antibodies specific to galactan, arabinan, XG and xylan showed that the lack of xylose wasn't due to a shortage of either of these polysaccharides. The cell wall polysaccharide fraction of both wild type and the mutant plants were treated with the enzyme XGA hydrolase, which cleaves xylogalacturonan molecules but not other pectic substrates. Nine new different oligosaccharides were present in the wild type plants after this treatment, while no changes were detectable in the insertional mutant, indicating that the decreased level of xylose was due to the absence of XGA. Based on these results, the protein encoded by the At5g33290 locus was named Xylogalacturonan Deficient 1 (XGD1) (Jensen et al., 2008). A XGD1-YFP construct was shown to colocalize with the STtmd-GFP GA marker fusion protein, providing further

evidence for the model in which pectins are, like hemicelluloses, synthesized in the GA and then transported to the cell wall via the secretory pathway (Jensen et al., 2008).

2.3 Secretory pathway and the deposition of pectins and hemicelluloses into the cell wall

The hypothesis that non-cellulosic cell wall polysaccharides are synthesized in the GA and transported to the cell surface in GA-derived secretory vesicles has been postulated already in the 1960's (Northcote and Pickett-Heaps, 1966). Nevertheless, the factors that underlie exocytosis and are thus responsible for proper cell wall composition remain largely unknown (Ischebeck et al., 2008). What is known in this field has been observed mostly in growing pollen tubes and root hairs; several such studies will be presented here.

Actin cytoskeleton seems to play an important role in determining the cell wall composition by regulating secretion and possibly by some other mechanisms (Vidali et al., 2001; Chen et al., 2007; Ischebeck et al., 2008). Pollen germination and pollen tube growth rates of *Picea meyeri* are reduced in a dose-dependent manner when the actin depolymerizing drug latrunculin B (LatB) is applied (Chen et al., 2007). When normal germinating pollen grains are stained with FM4-64, they mostly show a characteristic reverse V-like (inverted cone) pattern which marks secretory vesicles travelling towards the growing tip of the cell (Parton et al., 2001). When FM4-64 was applied to the pollen grains of *Picea meyeri* grown on medium containing LatB, this pattern wasn't apparent at all. Instead, the whole pollen tube was evenly stained with the dye, suggesting that vesicle trafficking was strongly disturbed in these cells (Chen et al., 2007). FT-IR spectrometry analysis of cell wall composition revealed that walls of the LatB treated cells contained significantly less esterified pectins, cellulose and protein. An immunolabeling assay with JIM5 and JIM7 antibodies also showed that the distribution pattern of esterified and de-esterified pectin within the growing pollen tube was changed dramatically by the application of LatB. All these results clearly indicate that F-actin depolymerization caused by the application of LatB severely disturbed secretory vesicle trafficking, which led to abnormalities in composition of the cell wall (Chen et al., 2007).

Vidali et al. observed reduced pollen germination and pollen tube growth in *Arabidopsis* after treatment with LatB and other actin depolymerizing agents. However, the

effect of actin depolymerization on the rate of cytoplasmic streaming was much weaker. The authors thus proposed that guiding secretory vesicles might not be the only function of actin cytoskeleton in the biogenesis of the cell wall in the pollen tube and that microfilaments may have additional functions in this process (Vidali et al., 2001).

Mur3 is a well described *Arabidopsis* fucose-deficient cell wall mutant .(Reiter et al., 1997). Tamura et al. have isolated an *Arabidopsis* mutant with defective actin cytoskeleton and endomembrane organization and named it *katamari1* (KAM1). Genetic and *in silico* analyses revealed that mur3 and KAM1 were allelic mutations of the gene At2g20370 (Tamura et al., 2005). Further experiments and analyses indicated that the KAM1/MUR3 encoded protein localizes to the GA. The protein's lumenal C-terminal part has glycosyl-transferase activity and its cytosolic N-terminal domain indirectly binds actin microfilaments. This led the authors to formulate a hypothesis that the KAM1/MUR3 is a multifunctional protein that's involved in the synthesis of cell wall material as well as in the organization of actin cytoskeleton. Furthermore, since actin cytoskeleton is an important factor in the exocytosis of GA-synthesized cell wall polysaccharides, KAM1/MUR3 might play a role in the crosstalk between the synthesis and transport of non-cellulosic cell wall components (Tamura et al., 2005).

$CdCl_2$ treatment caused Ca^{2+} gradient disruption and actin filament depolymerization and disorganization in *Arabidopsis* root hairs. FM4-64 labeling of $CdCl_2$ treated roots also showed that their vesicle trafficking was disturbed, and FT-IR analyses revealed altered composition of the cell wall. These results indicate that Ca^{2+} signaling probably plays an important role in the regulation of cell wall material secretion, either directly or by regulating the actin filaments which guide the secretory vesicles (Fan et al., 2011). Calcium is also known as a positive regulator of callose synthase activity (Fredrikson and Larsson, 1992).

While *Arabidopsis* double LOF mutants in phosphatidylinositol-4-phosphate 5-kinases 4 and 5 (PIP5K4 and PIP5K5) showed reduced pollen germination and defective pollen tube growth and morphology, pollen tubes of tobacco cells overexpressing either PIP5K4 or PIP5K5 exhibited multiple branching events and increased apical deposition of pectins. These results point out the possible role of PIP kinase signaling in the regulation of cell wall polysaccharides secretion (Ischebeck et al., 2008).

VHA-a1, a vacuolar V-ATPase VHA-a subunit isoform found preferentially in the Trans-Golgi Network (TGN), is necessary for proper trafficking of both endocytic and secretory vesicles in *Arabidopsis* root hairs and cell suspensions (Dettmer et al., 2006). However, whether the role of the V-ATPase is only to maintain specific pH values required for vesicle trafficking or whether it has different regulatory function remains unclear. These results also suggest that the secretory and endocytic pathways meet in the TGN, which serves also as the early endosome in plant cells, and that some endocytosed material might be carried back to the cell surface by exocytosis from this compartment (Dettmer et al., 2006).

Cell wall pectins have been indeed shown to be actively endocytosed in maize root apices (Baluska et al., 2002) and *Arabidopsis* seeds during imbibition (Pagnussat et al., 2012). These findings have led to the formulation of a hypothesis that some cell wall polysaccharides might be recycled in plant cells during various growth, morphogenetic and/or developmental processes (Baluska et al., 2002; Pagnussat et al., 2012).

3. Fourier Transform Infrared (FT-IR) spectroscopy in the study of plant cell walls

3.1 FT-IR principles and procedures

3.1.1 The physics behind FT-IR

Vibration spectroscopy is a study of molecular vibrations through changes in these vibrations caused by interaction with electromagnetic radiation (Trchová, 1995). Infrared (IR) spectroscopy is one of the two major methodological approaches to vibration spectroscopy, the other one being Raman spectroscopy (Trchová, 1995; Siebert and Hildebrandt, 2008).

Atoms within a molecule don't stand still; they vibrate in a certain direction, with a certain strength and frequency. FT-IR and Raman spectroscopy are called vibration spectroscopy methods because they are capable of detecting these molecular vibrations (Siebert and Hildebrandt, 2008). In FT-IR spectroscopy, this is possible because a molecular vibration of a chemical bond can absorb an IR beam when it has the same frequency; the probability of absorption depending on the polarity and strength of the bond, mass of the vibrating atoms and other intra- and intermolecular factors (Barth and Zscherp, 2002). Practically all polar molecular bonds can absorb IR radiation of some wavelength and thus all biomolecules can be studied by FT-IR spectroscopy (Barth, 2007).

An IR spectrometer is a device that registers and visualizes the absorbance and/or transmittance of IR radiation by the substance studied. The IR spectrum ranges approximately from 780 nm to 1000 μm and can be divided into three parts: *near-infrared* (780 nm - 2.5 μm), *mid-infrared* (2.5 μm – 50 μm) and *far-infrared* (50 μm – 1000 μm). In an IR spectrum plot absorbance is plotted against *wavenumber*, which is the inverse of the wavelength and is expressed in the unit cm^{-1} (Barth, 2007).

Dispersive spectrometers, which were commonly used up to the 1970s, used a monochromator and scanned the absorption/transmission for each wavelength separately. These machines were expensive and the process was very time-consuming: the collection of a single spectrum took up to several hours (Siebert and Hildebrandt, 2008). The Fourier transform (FT) is a mathematical expression of frequency as a function of time. In a FT-IR

spectrometer, time can be expressed as distance of the movable mirror (see next paragraph); the FT can then be modified to express frequency as a function of distance. The introduction of this principle to IR spectroscopy allowed scanning the absorption and/or transmission of multiple wavelengths simultaneously, thus reducing the spectra collection time enormously; this is called Felgett's advantage. The FT-IR spectrometers, unlike the older monochromator ones, have good resolution even at wide apertures (Jacquinot's advantage) and they don't need to be calibrated (Connes' advantage) (Trchová, 1995). Thanks to the above advantages, the FT-IR spectrometers have replaced the older dispersive ones completely over the past three decades (Siebert and Hildebrandt, 2008).

The principle component of most FT-IR spectrometers is a Michelson interferometer (Griffiths, 1975; Siebert and Hildebrandt, 2008), which consists of a beam splitter, a fixed mirror and a movable mirror (fig.4). The beam produced by the light source divides in two on the beamsplitter; one half is reflected back to the beamsplitter by a fixed mirror, the other half by a movable mirror that moves back and forth along the axis of the beam. Thanks to the moving mirror, a phase difference occurs between these two beams when they recombine at the beamsplitter which causes them to interfere (Griffiths, 1983).

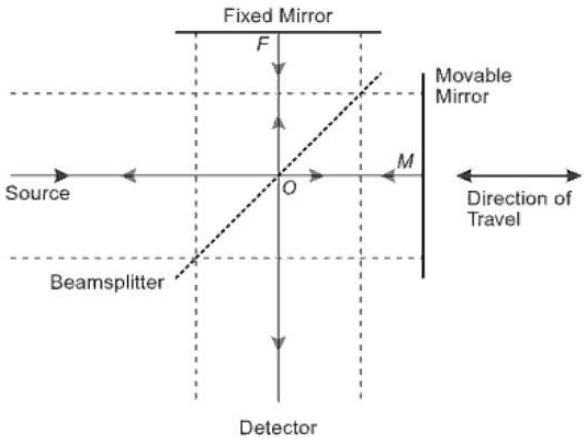


Figure 4
A diagram of the Michelson Interferometer. Courtesy of (Griffiths and De Haseth, 2007)

If the light source is monochromatic and the distance of the movable mirror from the beamsplitter is equal to that of the fixed mirror, there is no phase difference and the two beams interfere constructively upon recombination on the beamsplitter (this position of the mirror is called ZPD – Zero Path Difference). If the distance of the movable mirror changes by ¼ of the wavelength, the phase difference of the two beams is ½ wavelength and they interfere destructively (Griffiths, 1975). In reality though, the IR radiation source is polychromatic and emits photons of various wavelengths. All the beams interfere

constructively in ZPD; in all other movable mirror positions, some of them interfere constructively, some destructively (Trchová, 1995). The light wave leaving the beamsplitter is called the interferogram and it is the sum of all interfering beams of all wavelengths. The interferogram is detected by a detector and turned into a conventional IR spectrum by the FT (Siebert and Hildebrandt, 2008). If a substance is placed between the beamsplitter and the detector, it absorbs some of the radiance while letting another part pass. The sample‘s absorbance spectrum (fig.5) is expressed as the ratio of the background spectrum and the spectrum transmitted through the sample (Griffiths, 1983).

3.1.2 Statistical methods in FT-IR spectra evaluation

A FT-IR spectrum contains an enormous amount of data. When spectra of several samples are compared, the absorption at each wavelength represents one variable that needs to be included in the comparison. This makes it impossible to analyze FT-IR spectra by visual inspection; instead, multivariate statistical methods must be applied in order to obtain relevant information from a set of FT-IR spectra. Factor analysis, linear discriminant analysis and cluster analysis are most commonly used for this purpose (Mouille et al., 2003; Smith-Moritz et al., 2011).

Factor analysis is a method used to replace a large amount of variables in a data set by a lower amount of new variables without significant data loss. The basic concept of factor analysis is that most variables in a large data set are correlated with each other and can be explained by the existence of much fewer latent features (for example, different IR absorption of plant cell wall material at various wavenumbers can be explained by different contents of one or several cell wall polysaccharides). The aim of factor analysis is to explain as much of the original variability of a data set as possible by introducing a new set of independent variables that is as small as possible (Hebák and Hustopecký, 1987; Kopecký, 1998). Principal Component Analysis (PCA) is a factor analysis method widely used in FT-IR spectra evaluation (Chen et al., 1998; Smith-Moritz et al., 2011). In this method, the new variables are called Principal Component Scores (PCs). The PCs are ordered (PC1, PC2….) by the amount of variability of the data they explain. When two PCs are plotted against each other (usually, but not necessarily the first two PCs), clusters in the data set can be indentified (fig.5b,7). FT-IR data processed by PCA can either be evaluated directly by the inspection of PC plots or they can be further analyzed by linear discriminate and/or cluster analyses.

The aim of Linear Discriminate Analysis (LDA) is to project the variability of the data set in several variables into a single new variable (Hebák and Hustopecký, 1987). Variability is projected as distance on a single axis after LDA treatment, hence the word *linear* in its name. Several algorithms can be used to transfer variability from several variables into one, including normalized distance, Euclid distance and Mahalanobis distance, but only Mahalanobis distance respects both differential variability and correlation structure of the original data set (Hebák and Hustopecký, 1987). In FT-IR spectra analysis, Mahalanobis distance of the samples is typically calculated from the first few PCs obtained by PCA of the spectra (Chen et al., 1998; Smith-Moritz et al., 2011).

Cluster analysis is used to organize data into clusters so that the members of each cluster are more similar to each other than to any member of any other cluster (Hebák and Hustopecký, 1987; Kopecký, 1998). The similarity of samples is determined by one variable, in FT-IR spectra analysis typically the Mahalanobis distance (Mouille et al., 2003). Several clustering algorithms have been described, for example hierarchical clustering, centroid-based clustering, the Ward algorithm etc., each of which is advantageous in different applications. The result of cluster analysis can be visualized as a dendrogram (fig.5c,8)(Kopecký, 1998).

3.2 FT-IR spectroscopy applications in cell wall biology

FT-IR spectroscopy in combination with multivariate statistical analysis is a powerful tool for the study of the plant cell wall. Comparison of FT-IR spectra can reveal even very slight modifications in the polysaccharide composition of the cell wall. Moreover, different absorption values at certain wavenumbers can be linked directly to different amounts of specific cell wall components. Significant progress in assigning FT-IR absorption bands to individual polysaccharides has been made by Kacurakova et al. (tab.1) (Kacurakova et al., 2000).

No	Compound	(C–OH), (C–O–C), (C–C), ring					(Cl–H), ring			
1	Pectin	**1144s**	**1100vs**		1047, **1017vs**	953	896		857	835
2	Rhamnogalacturonan	1150	1122	**1070vs**	**1043vs, 989s**	951, 916	902		846	823
3	Galactan	1155	1134	**1072vs**	**1038vs**		893	883		
4	Arabinan	1141	1097	1070	**1039vs**	918	895			807
5	Arabinogalactan			**1074vs**	**1045vs**		897	868		808
6	Arabinogalactan	1139		**1078vs**	1043, 985			880	842	
7	Arabinogalactan (Type **II**)	1156		**1078vs**	1040	916	892	879		
8	Arabinogalactan (Type **II**)+Glucomannan (9:1, (w/w)	1146		**1066vs**	1034		896	872		809
9	Arabinogalactorhamnoglycan				**1049vs**	914			837	810
10	Xyloglucan	1153	1118	**1078vs**	**1041vs**	945	897			
11	Glucan	1151	1104sh	**1076sh**	**1041vs, 1026vs**	916			840	
12	Glucomannan	1150		**1092vs, 1064vs**	**1034vs**	941	898	872		814
13	Galactoglucomannan	1149		1064a	**1034vs, 960**	934	897	872		813
14	Arabinoglucuronoxylan + Galactoglucomannan	1161, 1151	1109	1070	**1038vs**		898	881		809
I	Pectin	1152	**1004vs**	1082, 1051	**1022vs**, 972		891		834	
II	GX	1147		1084	**1047vs**, 985		897			
III	Starch	1155	1110	1082	**1026vs**	931			850	
IV	Cellulose	1162	1120		**1059vs, 1033vs**	930	898			

Table 1 *FT-IR absorption band wavenumbers of cell wall polysaccharides (vs: very strong, s: strong IR band intensity; spectral data of compounds **I**–**IV** are taken from the literature). Courtesy of (Kacurakova et al., 2000)*

Several FT-IR spectroscopy applications in the identification of cell wall mutants and the study of interactions between cell wall polymers will be presented here. There are many more possible ways of using FT-IR based methods in cell wall biology, for example the study of effects of cellulose biosynthesis inhibitors on cell wall architecture (Alonso-Simón et al., 2011), chemotyping of plant tissues (Gorzsas et al., 2011) or cotton fibre development (Abidi et al., 2010). However, describing all of these applications exceeds the framework of this overview. Many FT-IR applications in the study of cell wall architecture and plant growth and development in general are covered in an excellent review (Dokken et al., 2005).

3.2.1 Sample preparation techniques

Most FT-IR spectrometers are capable of measurements in the transmission or reflectance mode. While the Attenuated Total Reflectance (ATR) technique is widely used in the analysis of proteins and other purified substances in solid and liquid state (Trchová, 1995; Barth, 2007), it is not usually applied to plant material, possibly because it is very heterogenous and doesn't have very good reflectance properties (Chen et al., 1998).

Direct transmission measurements of samples that have been handled minimally yield the best absorption spectra (Trchová, 1995). Dried hypocotyls of young seedlings can be measured directly on a FT-IR spectrometer attached to a microscope with good results. However, if these results should provide information about the composition of the cell wall, the seedlings must be grown in the dark on a sucrose-free medium so that they don't contain starch, whose absorption bands would otherwise overlap those of the cell wall polysaccharides (Mouille et al., 2003).

Several problems need to be addressed so that transmission FT-IR measurements of cell wall in leaves can be successful. The leaves can't contain any starch for the reason mentioned above; they must as well be depleted of chlorophyll, sugars and other small molecules, whose absorption bands would also spoil the signal of the cell wall polysaccharides. The starch problem is solved effectively by growing the plants in the dark 2-3 days before harvesting; chlorophyll and other molecules can be extracted from the harvested leaves by hot ethanol. FT-IR spectra collected from freeze-dried, starch-free, chlorophyll-depleted leaves provide good information about the composition of the cell wall. This sample preparation method has been proposed for measurements of small areas of leaves on a spectrometer with microscope accessory (Chen et al., 1998). However, light-grown and dark-grown (i.e. starch depleted) wild type *Arabidopsis* plants could be clearly divided when whole leaves are processed according to this protocol and their spectra are measured on a macroscopic spectrometer (fig.5), suggesting that this sample preparation technique could be used on a macroscopic level as well (Glanc, unpublished data).

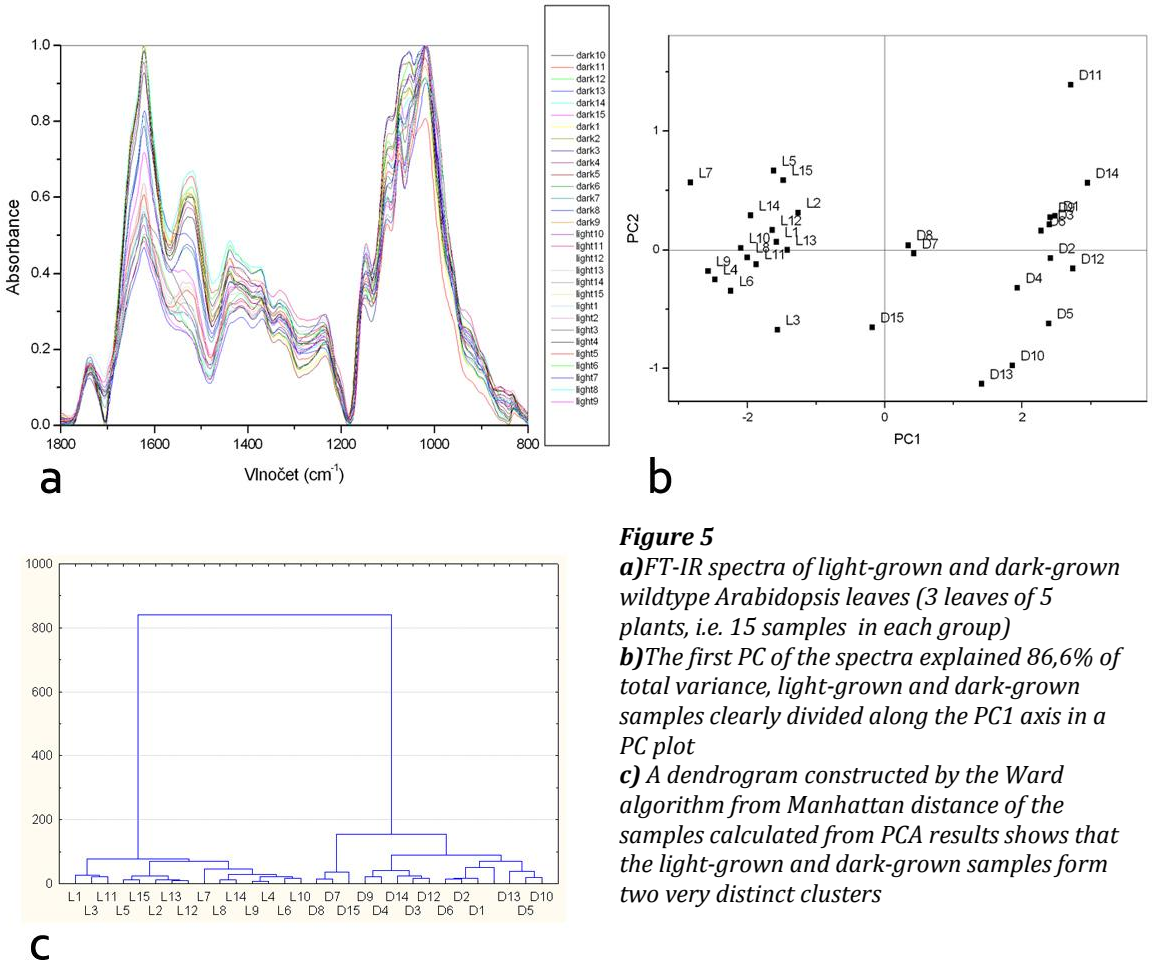


Figure 5
a)*FT-IR spectra of light-grown and dark-grown wildtype Arabidopsis leaves (3 leaves of 5 plants, i.e. 15 samples in each group)*
b)*The first PC of the spectra explained 86,6% of total variance, light-grown and dark-grown samples clearly divided along the PC1 axis in a PC plot*
c) *A dendrogram constructed by the Ward algorithm from Manhattan distance of the samples calculated from PCA results shows that the light-grown and dark-grown samples form two very distinct clusters*

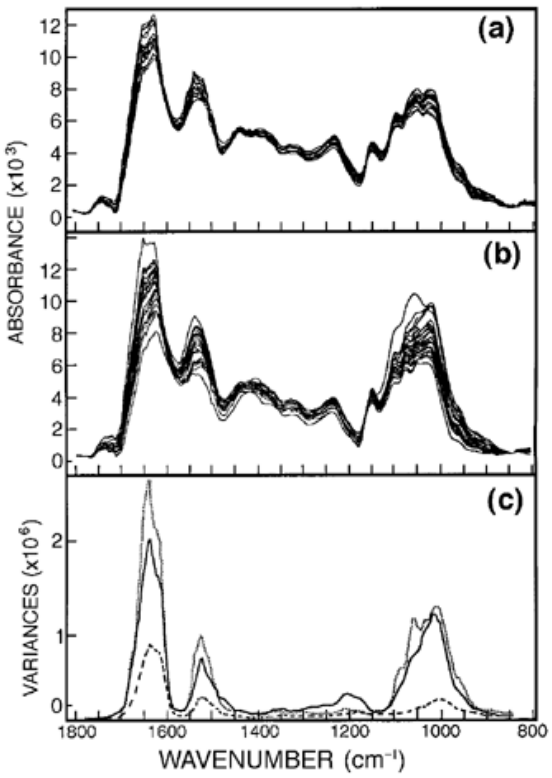
The cell wall material can also be extracted from the plant tissue and homogenized prior to FT-IR spectra collection. Thin sheets of isolated primary and secondary cell wall material have been shown suitable for FT-IR transmission measurements (Åkerholm and Salmén, 2001; Stevanic and Salmen, 2008). Some powder samples can be successfully measured when mixed with an optically neutral carrier, such as KBr, and pressed into transparent pellets (Trchová, 1995). However, this method hasn't proved suitable for measuring the spectra of isolated cell wall material (Glanc, unpublished data).

3.2.2 FT-IR spectroscopy in the identification and classification of cell wall mutants

Studies of cell wall deficient mutants are crucial for identification of genes and mechanisms responsible for proper cell wall biogenesis. Classic forward genetics approach is one way to obtain such mutants. In a forward genetics experiment, a large amount of samples is mutagenized. Subsequently, mutants with desired phenotype are identified,

isolated and used for further analyses. The efficiency of such a mutant screen depends on an effective and high-throughput screening method (Smith-Moritz et al., 2011). The teams of McCann and Höfte have come up with such screening methods for cell wall mutants based on Fourier Transform mid-Infrared spectroscopy (FT-MIR) (Chen et al., 1998; Mouille et al., 2003). Recently, a new screening method based on Fourier Transform near-Infrared (FT-NIR) spectroscopy was presented (Smith-Moritz et al., 2011).

McCann and his team based their screening method on the FT-IR transmission spectra of whole, chlorophyll depleted, freeze-dried leaves (Chen et al., 1998). After being baseline-corrected and area-normalized, these spectra were analyzed with PCA and LDA. This method successfully divided groups of wild-type *Arabidopsis* plants from those with characterized cell wall mutations; furthermore, it discriminated different mutants (*mur1* and *mur4)* from each other (fig.6,7). The method was then tested directly on an EMS-mutagenized population of 1000 flax plants. 59 plants were identified as putative cell wall mutants, chemical analyses of their cell wall sugar composition revealed that 36 of these 59 samples had actually altered amounts of at least one cell wall component (Chen et al., 1998).



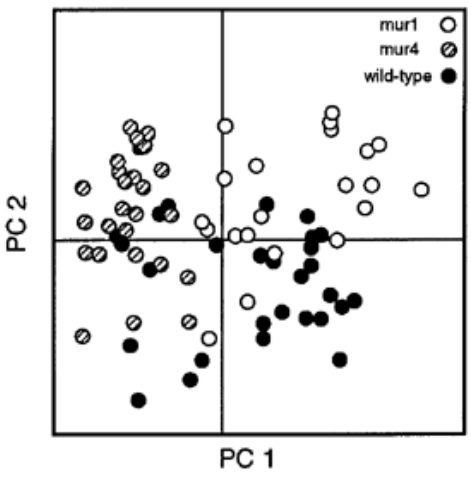


Figure 6 *(left)*
Representative wild-type (a) and mutant (b) baseline-corrected, area-normalized spectra and their calculated variance (c). Courtesy of (Chen et al., 1998)
Figure 7 *(above)*
A PCA plot showing that wild-type, fucose-deficient mur-1 and arabinose deficient mur-4 mutants can be divided based on their IR spectra. Courtesy of (Chen et al., 1998)

Höfte's team has chosen hypocotyls of 4-day-old etiolated *Arabidopsis* seedlings as material for transmission spectra collection (Mouille et al., 2003). They also treated the baseline-corrected, area-normalized spectra with PCA. Subsequently, they calculated the Mahalanobis distance between the spectra and used it to create a dendrogram of the samples (Mouille et al., 2003) An article focused solely on the statistical methods used in this work has been published (Robin et al., 2003). The final dendrogram (fig. 8) seemed biologically meaningful as it clustered all cellulose mutants in one branch. Moreover, it clustered mutations of comparable strength together; the method has thus also proved to be very useful in cell wall mutant screens (Mouille et al., 2003).

Very recently, the team of Miguel Vega-Sánchez has presented a new method to identify cell wall mutants, based on the NIR spectra rather than the MIR ones (Smith-Moritz et al., 2011). In their study, whole *Arabidopsis* leaf rosettes of previously characterized cell wall mutants were analyzed. PCA was applied to the area-normalized, baseline-corrected spectra, and Mahalanobis distance was calculated for individual samples, using the wild type *Col-0* plants as a reference. The mutants clearly clustered together in groups separate from each other and from the wild type (Smith-Moritz et al., 2011).

Next, several thousand mutagenized rice samples were analyzed and their Mahalanobis distance counted, using random samples as a reference. 5% of the samples with the greatest Mahalanobis distance were biochemically tested for cell-wall composition and 33% of these were shown to have altered content of at least one cell wall polysaccharide. The cell wall composition of part of these very same samples was then correlated with their spectra. Based on such calibration, cell wall composition of the other half of the samples was counted and showed 0,98 correlation coefficient with the experimental values (Smith-Moritz et al., 2011).

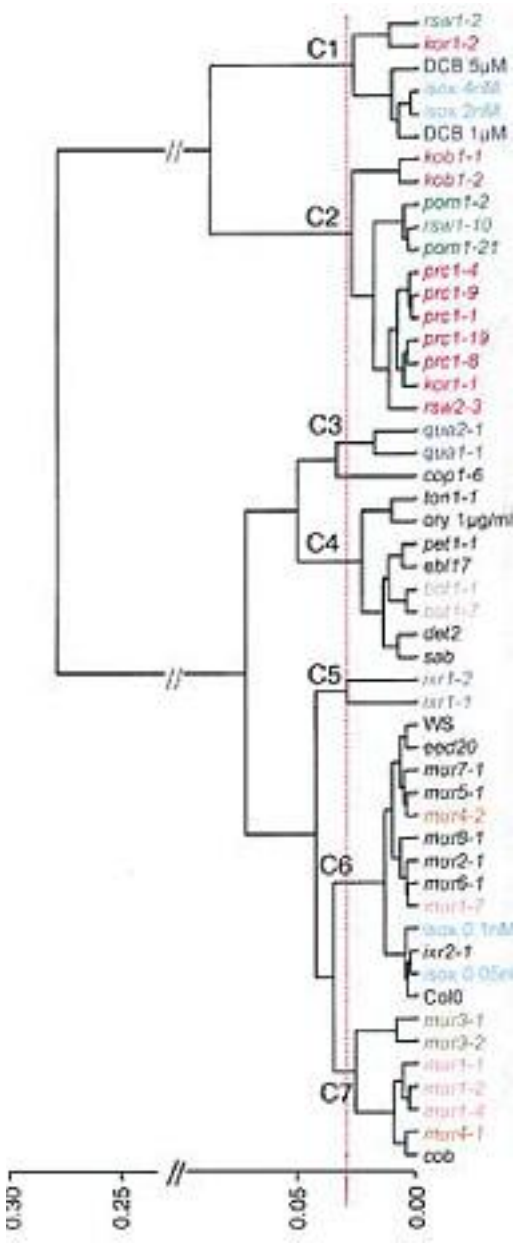


Figure 8
A dendrogram of the mutants used in the study obtained by hierarchical cluster analysis of their FT-IR spectra. Different mutant alleles of the same gene are written in the same color. C1-C7 are clusters of similar mutants, the cellulose-deficient mutants are in the C2 cluster. Courtesy of (Mouille et al., 2003)

In the next step, cell wall composition was predicted in all samples from the original population based on the comparison of their spectra with the calibration set. Putative mutants were selected, their cell wall composition tested experimentally. 60% of the putative mutants did indeed have altered content of at least one cell wall polysaccharide component (Smith-Moritz et al., 2011).The recently described method of Vega-Sánchez has two major advantages over the previous two: it requires almost no sample processing at all, and it can be performed on a regular FT-IR spectrometer that is not attached to a microscope (Smith-Moritz et al., 2011). However, the results are less informative. Another drawback of Vega-Sánchez's and McCann's methods is that they can't be applied to mutants that never grow leaves big enough for spectra collection, such as some cellulose deficient mutants. As one can see, all three FT-IR based methods for identification of cell wall mutants described above have certain advantages and drawbacks and neither one of them is universally better than the others. We can expect some alterations to these or completely new methods to be described in the future.

3.2.3 Dynamic two-dimensional FT-IR spectroscopy in the study of interactions between cell wall polymers

Dynamic FT-IR and two-dimensional FT-IR (2D FT-IR) are two advanced FT-IR techniques that have been used extensively over the past two decades in polymer research (Noda, 1989; Åkerholm and Salmén, 2001; Stevanic and Salmen, 2008). Dynamic and 2D FT-IR spectra, or a combination of the two, provide information on the changes that take place in a sample under mechanical stress and also on the interactions between polymers present in a sample. Also, they enhance the resolution in areas where overlapping peaks occur in conventional spectra (Åkerholm and Salmén, 2001). Dynamic FT-IR and 2D FT-IR are thus very useful in the study of properties and composition of plant cell walls.

The basic concept of 2D FT-IR spectroscopy is that the sample is exposed to an external perturbance during the collection of the spectrum, typically it is being repeatedly mechanically stretched in one direction. Knowing the frequencies of this strain and of the movement of the interferometer's movable mirror, two different spectra can be obtained: one where the sample is maximally stretched (in-phase spectrum) and the other where it is completely relaxed (out-of-phase spectrum) (Noda, 1989). These two spectra are then correlated with each other, resulting in a three dimensional plot, where the *x* and *y* axes represent the wavenumber scale of the in-phase and out-of-phase spectra and the *z* axis the resulting correlation intensity (fig.9) (Noda, 1989).

The spectrum of a sample under mechanical stress can be divided by the spectrum of the same sample without any perturbation. When absorbance is greater or smaller under the strain, the resulting spectrum shows positive or negative peaks, respectively. Such a spectrum is called dynamic. If dynamic spectra are plotted against each other instead of the static ones, the result is a dynamic 2D spectrum (Åkerholm and Salmén, 2001).

Åkerholm and Salmén used dynamic 2D FT-IR spectroscopy to study the interactions between cellulose and two hemicelluloses, xylan and glucomannan, in the secondary cell walls of tracheid fibers of spruce (Åkerholm and Salmén, 2001). They prepared thin sheets of extracted holocellulose pulp with different contents each polysaccharide. Based on multivariate analysis of static FT-IR spectra of these sheets, specific absorption bands were assigned to each polysaccharide (1735, 1600 and 1245 cm^{-1}

to xylan, 810 and 870 cm^{-1} to glucomannan and 1315, 1335 and 1430 cm^{-1} to cellulose) (Åkerholm and Salmén, 2001).

The samples were then exposed to a sinusoidal strain in a direction parallel or perpendicular to the cellulose fiber orientation in the sheets. Two sets of dynamic FT-IR spectra were collected for each strain direction, the IR source being polarized parallel or perpendicular to the strain direction, respectively. (Åkerholm and Salmén, 2001).

The dynamic spectra of the samples that were stretched perpendicularly to the fiber orientation had peaks at wavenumbers specific for all three studied polymers at both parallel and perpendicular IR polarization, meaning that all the polymers were affected by the strain. When the strain was applied parallel to the fiber orientation though, the results were quite different. The dynamic spectra had peaks at the cellulose-specific wavenumbers in both directions of IR polarization, while there were no peaks present in the xylan regions, suggesting the strain results in molecular changes of cellulose, but not xylan molecules. The glucomannan peaks appeared only when the IR was polarized perpendicular to the strain direction (and in this case, also the fiber orientation)(fig. 10a). This means that the glucomannan molecules are affected by the strain and also that they are oriented parallel to the cellulose microfibrils, which could be caused by interactions between cellulose and glucomannan. 2D correlation of the dynamic spectra provided further evidence for this hypothesis, as the dynamic 2D FT-IR spectrum had crosspeaks between all the bands specific for cellulose and glucomannan (fig.10b)(Åkerholm and Salmén, 2001).

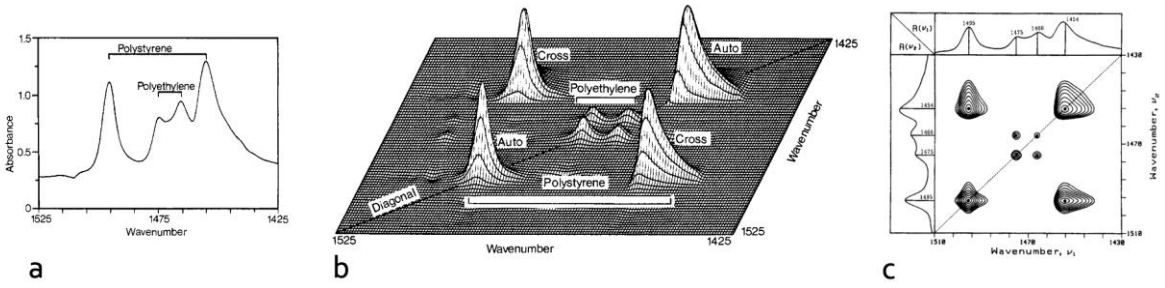


Figure 9 *A static IR absorption spectrum of a mixture of polystyrene and polyethylene (a); 2D IR correlation spectrum of the same sample under tensile oscillatory strain with a frequency of 23 Hz and amplitude of ca. 0,1% sample length in Fishnet representation (b) and contour map representation (c). Courtesy of (Noda, 1989)*

Stevanic and Salmen were interested in the differences between interactions of polymers in the primary and secondary cell walls (Stevanic and Salmen, 2008). For this purpose, thin sheets of isolated primary cell wall material of spruce were prepared and dynamic FT-IR spectra were collected according to (Åkerholm and Salmén, 2001). These spectra contained peaks at wavenumbers characteristic for all major cell wall components at both 0° and 90° IR polarization: cellulose around 3240, 3270 and 1435 cm^{-1}, lignin around 1270 and 1510 cm^{-1}, hemicelluloses around 1735 and 1595 cm^{-1} and also peaks in a wide region between 1665-1555 cm^{-1}, which contain signal from proteins as well as pectin (Stevanic and Salmen, 2008). These results indicate that the polymers of the primary cell wall constitute a very tight network where all the components interact with each other and all of them respond to a mechanical stress applied in any direction. This is very different from the situation in the secondary cell wall, where the major constituent cellulose interacts only with some other components and is much more affected by mechanical stress than other polysaccharides (Åkerholm and Salmén, 2001; Stevanic and Salmen, 2008).

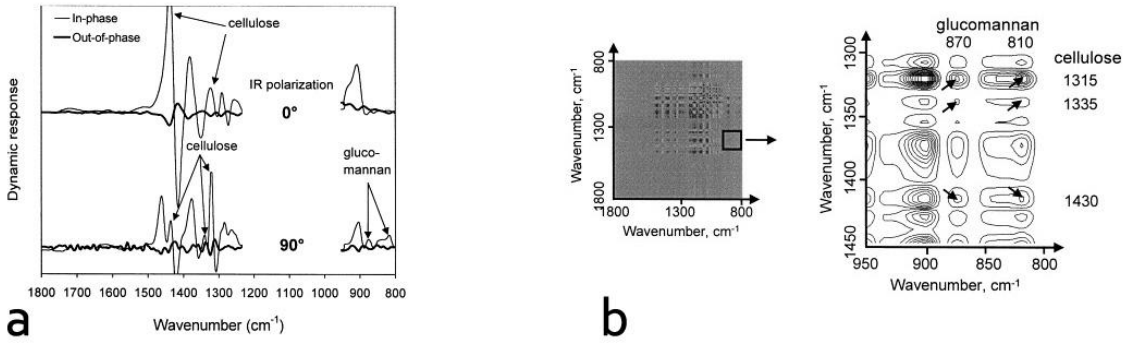


Figure 10
a) *Dynamic FT-IR in-phase and out-of-phase spectra of the sample under low amplitude sinusoidal strain parallel to the fiber orientation, with IR source polarized parallel (0°) and perpendicular (90°) to the strain direction. Peaks at cellulose-specific wavenumbers are present in both polarization modes, glucomannan peaks are significant only at 90° polarization mode and there are no xylan peaks visible at either mode*
b) *Dynamic 2D FT-IR spectrum obtained by plotting the two in-phase spectra mentioned above against each other. Crosspeaks at all wavenumbers specific to cellulose and glucomannan suggest the existence of strong interactions between these two polymers. Courtesy of (Åkerholm and Salmén, 2001)*

4. Outlook

Some genes that take part in the biogenesis of the plant cell wall have been identified and their protein products characterized as presented in this work. However, the vast majority of mechanisms involved in cell wall biogenesis remain unknown. For illustration, the genome of *Arabidopsis* encodes over 730 putative glycosyltransferases and glycosyl hydrolases, as well as several hundred different proteins that are thought to have a function in cell wall biogenesis (Somerville et al., 2004).

The experiments and results described earlier in this work demonstrate that various FT-IR spectroscopical techniques provide a powerful tool for the study of various aspects of plant cell wall biology, including chemical composition, interactions between polymers and mutant identification. Nevertheless, only few groups have attempted to use FT-IR spectroscopy to unravel the mechanisms by which exocytosis affects biogenesis of the cell wall (Chen et al., 2007; Fan et al., 2011).

There is strong evidence that the secretory pathway plays a key role in the plant cell wall biogenesis. However, only several studies addressing the effects of secretory pathway malfunctions on cell wall synthesis and composition have been published (Tamura et al., 2005; Ischebeck et al., 2008). If we want to fully understand the role of the secretory pathway in cell wall biogenesis regulation, the effects of many more vesicle trafficking mutations and pharmacological treatments on cell wall composition need to be examined, and FT-IR spectroscopy seems to be the ideal tool for such experiments.

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