Synthesis of Glucuronoxylan in Higher and Lower Plants – Is There Conservation of the Enzymatic Machinery?

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Cover: *Physcomitrella* gametophores displaying PpGT47D:GUS staining.
(photo: E. Hörnblad)

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Abstract

The hemicellulose glucuronoxylan (GX) is the second most abundant polysaccharide after cellulose in the secondary cell walls of angiosperms and contributes significantly to the properties of wood as raw materials. Despite this, very little is known about the biosynthesis of hemicellulose. The thesis is based on studies performed in *Physcomitrella patens*, *Arabidopsis thaliana*, and *Populus* plants with the aim to increase the understanding of glucuronoxylan synthesis, and with main focus on the role of the *IRX10* gene family.

A number of genes have proven to be involved in GX biosynthesis in *Arabidopsis*, namely *IRREGULAR XYLEM* (*IRX*) *9*, *IRX14*, *IRX8*, *FRAGILE FIBER* (*FRA*) *8* and *PARVUS*. This thesis presents two new genes, *IRX10* and its close homolog *IRX10-LIKE (L)*, which can now be added to the group of presumed GX biosynthesis genes. In addition, identification and characterisation of three homologs (*IRX9-L*, *IRX14-L* and *F8H*) of previously identified GX synthesis genes is described.

Complementation experiments and morphological studies confirmed redundancy between the *IRX9* and *IRX9-L*, *IRX14* and *IRX14-L*, and the *FRA8* and *F8H* pairs of genes in *Arabidopsis*. A bioinformatics based approach has led to the identification of four putative homologs in *Populus trichocarpa* (*PtGT47A-1*, *PtGT47A-2*, *PtGT47D-1*, and *PtGT47D-4*) of *AtIRX10*, and one putative homolog from *Physcomitrella* (*PpGT47D*). Experiments further confirmed functional conservation between the *Arabidopsis* (IRX10) and *Populus* GT47A proteins, and partial functional conservation between the *Populus* GT47D, the *Physcomitrella* GT47D and *Arabidopsis* IRX10 proteins.

The work supports the existence of an *Arabidopsis* GX backbone biosynthesis complex in which AtIRX10 and AtIRX10-L form an essential component together with AtIRX9 and AtIRX14. It also suggests that parts of the GX biosynthesis machinery are conserved across embryophytes. Finally, work presented in this thesis indicates that the *IRX10* gene family has gone through a subfunctionalisation event during the evolution.

Keywords: Secondary cell wall, glucuronoxylan, *Populus*, *Physcomitrella*, *Arabidopsis*, *IRX10*, *IRX10-L*, *IRX9*, *IRX9-L*, *IRX14*, *IRX14-L*, GT47A, GT47D, glycosyltransferases

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”Gutta cavat lapidem, non vi seds aepe cadendo”(”Trägen vinner” typ☺)

Publius Ovidius Naso

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wu, A., Rihouey, C., Seveno, M., **Hörnblad, E.,** Singh, S. K., Matsunaga, T., Ishii, T., Lerouge, P. and Marchant A. (2009). The *Arabidopsis* IRX10 and IRX10-LIKE glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell wall formation. *The Plant Journal*, **57**, 718-731.

II Wu, A., **Hörnblad, E.,** Voxeur, A., Gerber, L., Rihouey, C., Lerouge P. and Marchant A. (2010). Analysis of the *Arabidopsis IRX9/IRX9-LIKE* and *IRX14/IRX14-LIKE* Pairs of Glycosyltransferase Genes Reveals Critical Contributions to Biosynthesis of the Hemicellulose Glucuronoxylan. *Plant Physiology*, **153**, 542-554.

III **Hörnblad, E.,** Wu, A., Gorzas, A., Gerber, L. and Marchant A. The *Populus GT47A-1* gene encodes a functional ortholog of the *Arabidopsis* IRX10 and IRX10-Like proteins supporting a common mechanism for glucuronoxylan biosynthesis between woody and herbaceous plants (manuscript).

IV **Hörnblad, E.,** Ulfstedt, M., Ronne, H. and Marchant A. An investigation into the role of the *Physcomitrella patens* GT47D protein (manuscript).

The contribution of Emma Hörnblad to the papers included in this thesis was as follows:

I Contribution – 10%. Significant intellectual contribution to discussions of the results and data analysis. Analysis of the monosaccharide sugar composition of wild-type and mutant plants. Input into the writing of the paper.

II Contribution – Experimental 15%. Analysis of the monosaccharide sugar composition of wild-type and mutant plants. Significant intellectual contribution to discussions of the results and data analysis. Input into the writing of the paper.

III Contribution – 95%. Carried out the study, designed experiments and analysed data obtained. Any collaborative work was undertaken with the close involvement of Emma Hörnblad. She wrote the manuscript.

IV Contribution – 90%. The majority of work in this manuscript has been either carried out by Emma Hörnblad or in close collaboration with colleagues in Uppsala. All experiments were designed and analysed by Emma Hörnblad and the manuscript was written by her.

Abbreviations

AA	alditol acetates
AGPs	arabinogalactan proteins
AIR	alcohol insoluble residues
Api	apiose
Api*f*	apifuranosyl
Ara	arabinose
Ara*f*	arabinofuranosyl
AT	acetyltransferase
At	*Arabidopsis thaliana*
ATs	acetyltransferases
AXs	arabinoxylans
CesA	cellulose synthase
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectroscopy
F8H	FRAGILE FIBER 8 HOMOLOG
FRA8	FRAGILE FIBER 8
FT-IR	Fourier transform infrared spectroscopy
Fuc	fucose
Fuc*p*	fucopyranosyl
Gal	galactose
GalA	galacturonic acid
Gal*p*	galactopyranosyl
Gal*p*A	galactopyranosyluronic acid
GAXs	glucoarabinoxylans
GC/MS	gas-chromatography mass spectroscopy
GHs	Glycoside hydrolases
Glc	glucose
GlcA	glucuronic Acid
Glc*p*	glucopyranosyl

Glc*p*A	glucopyranosyluronic acid
GRPs	glycine-rich proteins
GTs	glycosyltransferases
GUT1	GLUCURONYLTRANSFERASE 1
GUX	GLUCURONIC ACID SUBSTITUTION OF XYLAN
GX	glucuronoxylan
GXs	glucuronoxylans
HGs	homogalacturonans
HP-SEC	high-pressure size-exclusion chromatography
HRGPs	hydroxyprolinerich glycoproteins
IRX	IRREGULAR XYLEM
LCCs	lignin-carbohydrate complexes
Man	mannose
Man*p*	mannopyranosyl
Mbp	Megabase-pairs
MeGlcpA	methyl glucopyranosyluronic acid
miRNAi	microRNA interference
NDP	nucleotidediphosphate
NMR	nuclear magnetic resonance
Np	*Nicotiana plumbaginifolia*
PEG	polyethylene glycol
Pp	*Physcomitrella patens*
PRPs	proline rich proteins
Pt	*Populus trichocarpa*
Ptt	*Populus tremula x tremuloides*
qPCR	quantitative PCR
RG	rhamnogalacturonan
Rha	rhamnose
Rha*p*	rhamnopyranosyl
RNA	ribonucleic acid
RNAi	RNA interference
RWA	REDUCED WALL ACETYLATION
siRNA	small interfering RNA
T-DNA	transfer DNA
TMS	trimethylsilyl
WAKs	wall-associated kinases
Xyl	xylose
Xyl*p*	xylopyranosyl

1 Introduction

Throughout evolution, plants have constantly developed novel traits that have enabled them to colonise new niches or to outcompete other existing species. The development of vascular tissues, i.e xylem and phloem, for transport of water and nutrients is one of those traits, since it has allowed some plants to increase in height and efficiently utilise resources that are less available for the smaller non-vascular plants (Jung & Park, 2007; Sarkar *et al.*, 2009). The immense strength and transport capacity of well-developed vascular tissues makes it for example possible for some tree species to grow to a height of more than impressive 100 m. This would be impossible if the only type of water and soluble nutrients transport was trough diffusion.

The xylem and phloem cells of the vasculature form continuous interconnected tubular networks that are able to efficiently transport water and nutrients. The xylem cells are reinforced via the formation of secondary cell walls which provide physical support and improve the ability of the plant to transport water and nutrients to peripheral tissues (Jung & Park, 2007). The secondary cell wall is deposited inside the primary wall before the xylem cells undergo cell death and lose their cell contents. The mature xylem is the predominant structure in gymnosperm and angiosperm trees.

Similarities and differences between higher and lower plants can be used for comparisation in order to find out the evolutional history of certain traits and to figure out how certain biological processes work in general among plants. Lower plants are traditionally called non-vascular plants, but in fact primitive vascular systems can be found in some non-vascular plants. Specialised cells for water and nutrition transport found in bryophytes possess no secondary cell walls, and the conducting properties are less pronounced than in the vasculature of higher plants. Despite this, most components making up cell walls of the xylem and phloem of vascular plants are present in cell walls of many mosses.

This thesis is based on studies of non-vascular *Physcomitrella patens* moss (hereafter referred to as *Physcomitrella*), herbaceous *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) plants, and woody *Populus* trees in order to figure out more about how plants use the *IRX10* family of genes for the biosynthesis of the cell wall polysaccharide glucuronoxylan, a major component of the angiosperm secondary cell wall.

2 The Plant Cell Wall

Plants are multicellular organisms which are made up by specialised cell types that form morphologically distinct tissues. Unlike cells of animals and protozoans, but similar to i.e. prokaryotic bacteria, fungal cells, non-green algae and some archaebacteria, all plant cells have a cell wall.

The plant cell walls are mainly comprised of polysaccharides. This contrasts with the cell walls of fungi which are predominantly made up of chitin, the cell walls of non-green algae and eubacteria which contain peptidoglycans, and the cell wall of some archaebacteria which have glycoproteins as major components (Sarkar *et al.*, 2009). Evolutionary, the ability to synthesise new cell wall components, and to synthesise certain cell wall components more efficiently, have contributed to the formation of new species (Jung & Park, 2007). Likewise, many features of the plant specific cell walls have most likely played important roles in evolutionary key events such as multicellularity and the colonisation of land by plants (Popper *et al.*, 2011). A cladistic summary of the occurrence of cell walls is presented in Fig. 1.

2.1 The Primary Cell Wall

Common for all plant cells, is that they have primary cell walls mainly composed of structural proteins and cellulosic, pectic and hemicellulosic polysaccharides in differing relative amounts dependent on the cell type and species investigated. The components of the primary cell wall make up a mesh-like structure in which they are linked together by covalent and non-covalent linkages (Fig. 2A), that withstands the turgor pressure and provides rigidity to the cell. The same basic mesh-like architecture can be seen in all phylogenetic groups of plants (O'Neill & York, 2003; Sarkar *et al.*, 2009).

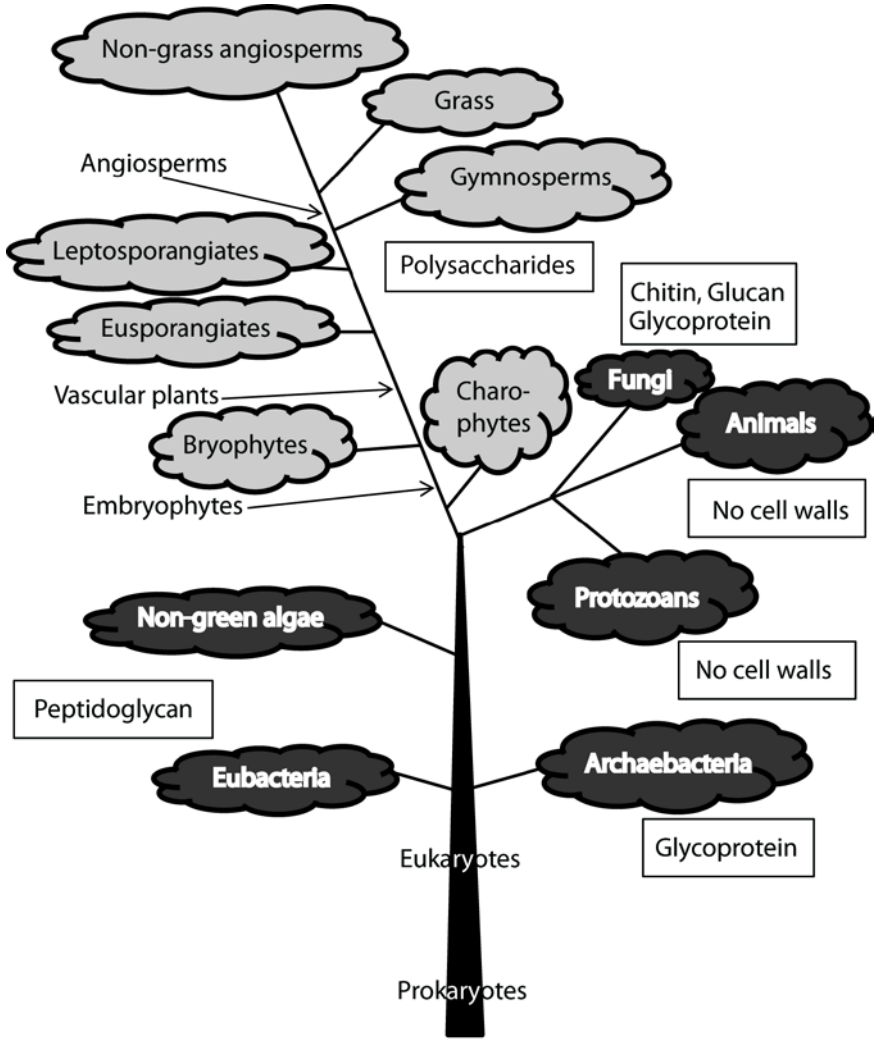

Figure 1. Phylogenetic tree showing the plant kingdom in light grey. The lengths of the branches are not in proportion to the degree of relation between the subgroups. Modified from Sarkar et al. *(2009).*

There are two main types of primary cell walls in angiosperms; type I found in dicotyledons and many monocotyledons, and type II found specifically in the monocotyledonous grass species. The type I primary cell wall typically contains about 20-30% cellulose, 15-30% hemicelluloses and 30-40% pectins. Typical for the type II primary cell wall is the relatively low amount of pectins (<10%) and the higher proportion of hemicellulosic xylan (20-40% compared to <5% in type II primary cell walls) and mixed-linked glucan (10-30% compared to absent or varying if present in type II primary cell walls; Carpita

& Gibeaut, 1993; Carpita, 1996; O'Neill & York, 2003). In some of the water conducting cells, lignin or lignin-like polymers are later on added to the primary cell wall, impregnating the tissue (Mishler & Churchill, 1984; O'Neill & York, 2003; Sakakibara *et al.*, 2003; Sarkar *et al.*, 2009).

The primary cell wall is first deposited in the cell plate of the dividing cell and then spreads toward the already existing primary cell wall of the mother cell to completely surround the plasma membranes of both daughter cells. The cell plate develops into the pectin rich middle lamella found between the cells, and which cements the cells together. The deposition of the primary cell wall occurs until the cell reaches its final size (Doblin *et al.*, 2003).

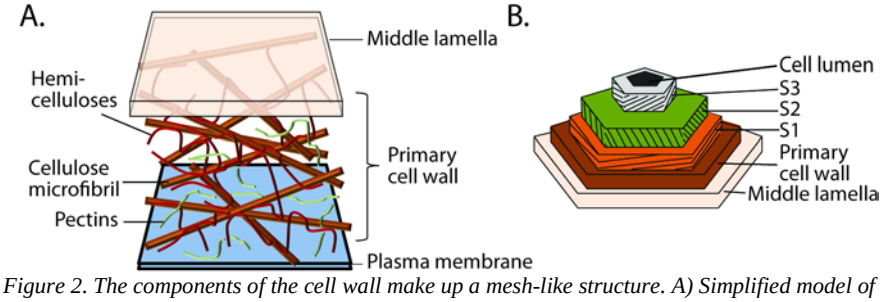

Figure 2. The components of the cell wall make up a mesh-like structure. A) Simplified model of the primary cell wall. B) The secondary cell wall is made by the S1, S2 and S3 layers. Figure A modified fromUnited States Department of Energy Genome Programs (genomics.energy.gov) and Fiugure B modified from (Cote, 1967).

2.2 The Secondary Cell Wall

Tracheids, vessels and fibers form secondary cell walls which are deposited on the inner face of the existing primary cell walls, and provide additional strength and rigidity to the cells. Secondary cell walls are like the primary made of polysaccharides. The biggest proportion is cellulose, as in the case for the primary cell wall, but the secondary cell wall contain very small if any amounts of pectins, and high proportions of hemicellulosic xylans (angiosperms) or mannans (gymnosperms). The water conducting properties are further improved by lignification, in which the deposition of lignin adds strength and impermeability to the secondary cell wall (Zhong & Ye, 2009).

The secondary cell wall is deposited as a similar type of mesh-like structure as the primary (Fig. 2A); although in many species it is organised in layers which are defined by the orientation of cellulose microfibrils. Most secondary walls have three layers called S1, S2 and S3 (Fig. 2B; Zhong & Ye, 2009).

The ability for plant cells to make secondary cell walls is considered one of the most important evolutionary landmarks for vascular plants since the formation of secondary cell walls within the vascular tissues allows the cells to

withstand high pressure and facilitates their function in water transport (Zhong & Ye, 2009).

2.3 Wood Development

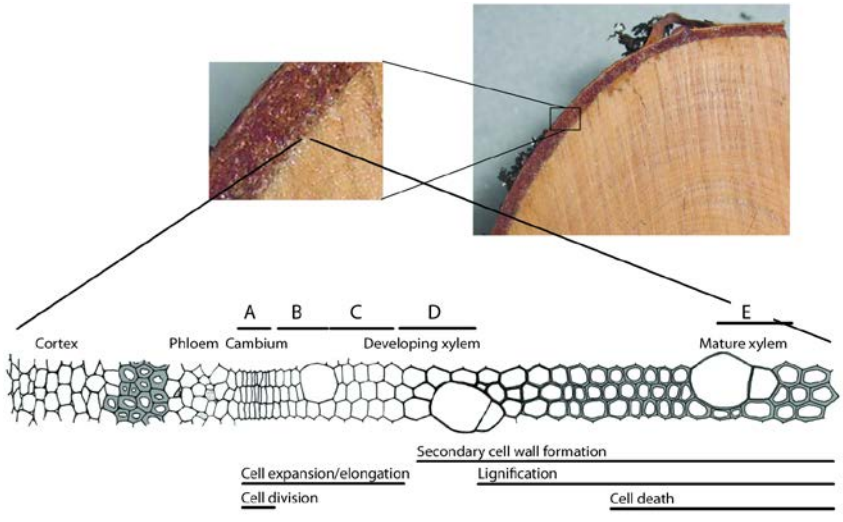

Figure 3.The stem displays a developmental gradient with the most mature secondary xylem cells close to the center of the stem (most to the right in the picture), and newly formed xylem cells close to the cambial region of the stem (zone A and B in the picture). Figure credit Ellinor Edvardsson (2010). Modified from Schrader et al. *(2004).*

The vascular stem develops in two steps; first by formation of the primary vasculature which is derived from cells in the apical meristem, and then by the formation of secondary vasculature which thickens the diameter of the stem and is formed by cells of the vascular cambium (Jung & Park, 2007). The secondary xylem is deposited in several layers of cells, with the old and mature cells furthest in to the middle of the stem and the newly formed xylem cells close to the meristem. In this way, the stem can be said to display a developmental gradient (Fig. 3) starting in the cambium with the dividing meristematic cells, and then inwards passing expanding cells with primary cell wall, fully elongated cells which deposit secondary cell wall, secondary cell wall containing cells which initiate cell death and which deposit lignin, and finally mature, dead and lignified secondary xylem cells (Hertzberg *et al.*, 2001a; Schrader *et al.*, 2004; Zhang *et al.*, 2011). The mature xylem cells are the main components of the structure in trees referred to as wood.

2.4 Biosynthesis of the Cell Wall

The cell wall contains a number of enzymatic proteins such as glycoside hydrolases (GHs), proteases, glycosidases, peroxidases, acetyltransferases (ATs) and esterases which are vital for remodeling and maintenance of cell wall integrity under growth, stress, and environmental changes (Fry, 1995; O'Neill & York, 2003). However, the biosynthesis of the hemicelluloses and pectins takes place in the Golgi apparatus and is catalyzed by groups of enzymes called glycosyltransferases (GTs), which facilitate the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming the glycosidic bonds of the polysaccharides (Sinnott, 1990). Cellulose synthesis is also carried out by GTs, but the cellulose synthesising enzymes are arranged in a terminal complex at the surface of the plasma membrane where it synthesises the polysaccharide directly into the cell wall outside the cell (Carpita, 2011; Cheng *et al.*, 2011). Hemicelluloses and pectic polysaccharides are transported from the Golgi to the plasma membrane where they are excreted outside the cell and form the mesh-like cell wall structures together with cellulose (Riese *et al.*, 2003). The cell wall is further strengthened by structural proteins, and in some species and cell types, by lignification where phenolic compounds including lignins or lignans are incorporated into the mesh of polysaccharides (Zhong & Ye, 2009).

2.4.1 Glycosyltransferases

All GTs include a catalytic site where the enzymatic activity occurs, a substrate binding site (sometimes associated with a carbohydrate binding module) and an acceptor binding site. These three features are dependent on the amino acid sequence and the resulting 3D structure of the protein which thus have a major impact on the specificity and efficiency of the enzyme (Lairson *et al.*, 2008).

Depending on their predicted 3D structure, GTs are divided into two major groups or folds; GT-A and GT-B (Coutinho *et al.*, 2003; Lairson *et al.*, 2008). The GTs are further divided into families depending on their sequence similarity, but as GTs can contain sequences or modules characteristic for several families the nomenclature does not always follow this rule.

In order to fully understand cell wall biosynthesis, major efforts have been undertaken to elucidate the enzymatic function of cell wall related GTs. The proteins within one family can generally be thought to have an enzymatic activity that is either inverting or retaining, depending on the stereochemical outcome of the product. GTs within one family are thought to share catalytic mechanism, but this is a rule with many exceptions as even differences in single amino acid positions within the active site can drastically change the enzymatic properties of the enzyme, and the barrier between inverting and

retaining mechanisms for single GTs for example have turned out to be small (Lairson *et al.*, 2008). In addition, single amino acid substitutions between closely related sequences can give rise to enzymes with very different 3D structure, which affects both substrate and acceptor binding properties. Furthermore, some of the plant GTs can act promiscuously and have several endogenous functions. Consequently, many GT families have been shown to be polyspecific and hence the 3D structure, catalytic activity and substrate binding properties must always be confirmed by functional studies (Breton & Imberty, 1999).

2.4.2 Cell Wall Precursors

Most cell wall polysaccharides are synthesised in the Golgi apparatus by GTs, and then transported to the cell wall to be incorporated into the growing extracellular cellulosic network (Scheible & Pauly, 2004). GTs uses nucleotidediphosphate (NDP) -conjugated monosaccharides, also called NDP-sugars, as donor substrates. NDP-sugars are carbon compounds derived ultimately from glycerol compounds as a result of photosynthesis, and are used for many basic functions in the cell other than for cell wall synthesis, such as glycosylation of proteins and starch biosynthesis (Scheible & Pauly, 2004; Sharples & Fry, 2007). The NDP-sugars and their precursors are metabolically linked and competing synthesis pathways are often present (Fig. 4). Moreover, the pathways are to a certain extent reversible, allowing the plants to remobilise components according to need. Thus the sugar metabolism in plants is complex and not surprisingly the exact nucleotide sugar substrate is yet to be demonstrated for a number of cell wall polysaccharides. Products labeled with radioactivity deriving from *Arabidopsis* cells grown in the presence of radioactivity labeled carbon and nitrogen sources have been useful to elucidate the metabolic pathways of NDP-sugars in plants (Sharples & Fry, 2007).

Lower plants share many basic primary metabolic enzymes with higher plants, suggesting a high degree of conservation in sugar nucleotide and polysaccharide biosynthesis within the plant kingdom (Salerno & Curatti, 2003; Thelander *et al.*, 2009)

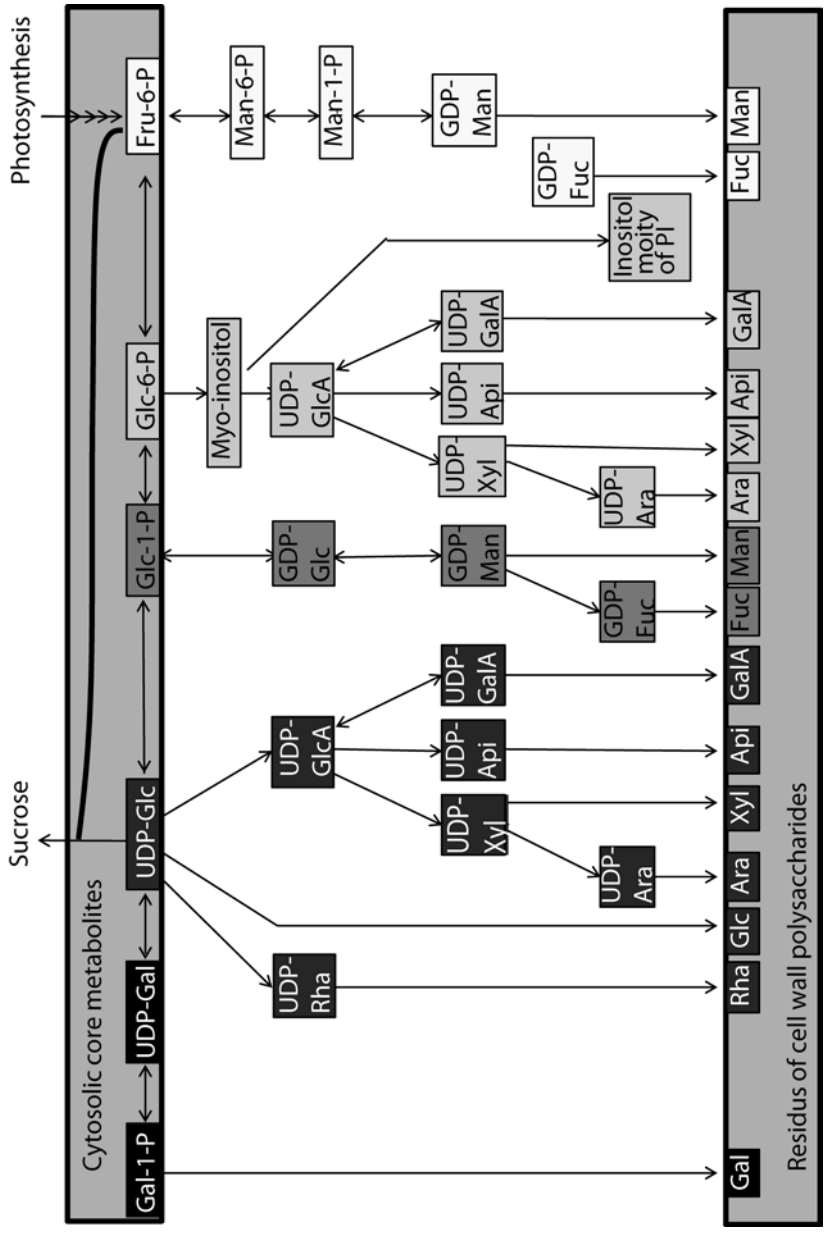

Figure 4. NDP-sugars are substrates used by GTs to build the cell wall. Many reactions are reversible as shown in the Figure. Modified from Sharples and Fry. (2007).

2.5 Components of the Cell Wall

Polysaccharides are the main components of the plant cell walls. Many of the major cell wall polysaccharides are present in the cell walls of all land plants,

although in different relative amounts(Moller *et al.*, 2007). Further, the exact composition of the components has been showed to differ between species and taxas (Peña *et al.*, 2008; Kulkarni *et al.*, 2012). These differences are often thought to be the result of divergent evolution, but in some cases the same or similar componets are thought to be the result of convergent evolution, i.e. same component have aroused several times in different organisms during the evolution. This is e.g. the case for lignin or lignin-like coumpounds that are thought to have arisen at least two times; once in red algae and once in embryophytes, and then possibly a third time in charophycean green algae (Martone *et al.*, 2007; Sorensen *et al.*, 2010).
The occurrence of the major cell wall components in any form in land plants and the closest relative to land plants, charophycean green algae, is concluded in Fig. 5.

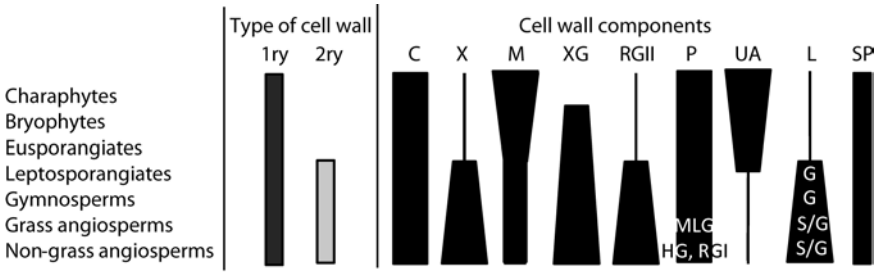

Figure 5. The primary and secondary cell wall components of the major phylogenetic groups of plants. C=cellulose , X=xylans, M=mannans, XG=xyloglucans, P=Pectins, MLG= mixed-linked glucans, HG=homogalacturonans, RG=Rhamnogalacturonan, UA=uronic acids, P=structural protein,s S=syringyl units, G=guaicyl units. Modified from Sarkar et al. (2009; Popper, 2008; Popper & Tuohy, 2010).

2.5.1 Cellulose

Cellulose is the mostly used cell wall component for industrial purposes such as pulp and paper production and cotton production, and it is the main source of energy in biofuels. Thus, cellulose has long been considered the most important component of the cell wall and therefore has been the subject of much investigation. Cellulose is a non-branched polysaccharide composed of β-D-(1→4)-glucopyranosyl (Glc*p*) units, which are joint together by a set of GT2 cellulose synthases (CesAs) catalysing the transfer of UDP-D-Glc*p* units to growing chains of β-D-(1→4) glucan (Cosgrove, 2005).

In most organisms cellulose is synthesised by CesA proteins. In land plants, CesA proteins, sometimes together with other proteins, form cellulose synthesising complexes referred to as rosette terminal complexes (Fig. 6). The rosette complexes are situated at the plasma membrane of the cell and deposit

the cellulose outside the cell. Each complex is thought to make 24-36 single β-D-(1→4) glucan chains that together form a microfibril which is approx. 2.4-3.8 nm in diameter (Carpita, 2011). The microfibrils are evenly distributed in the primary cell wall as a mesh around the cell (Fig. 2A). In secondary cell walls the cellulose microfibrils are highly organized and changes in the orientation of cellulose microfibrils during their deposition lead to the formation of distinctive layers (Fig. 2B; Zhong & Ye, 2009).

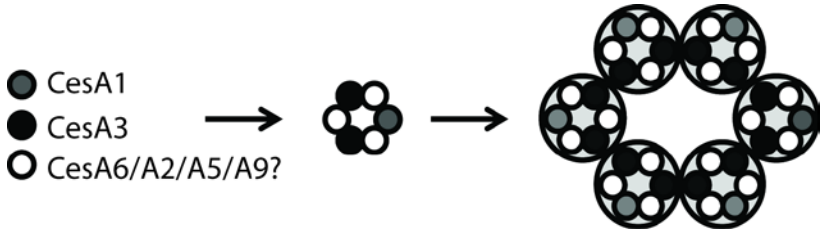

Figure 6. A model of the Arabidopsis *six units cellulose rosette terminal complex. Each unit is made up by six CesA enzymes; CesA1, CesA3 and CesA2, CesA5, CesA6 or possibly CesA9 synthesising the primary cell wall cellulose. A similar complex is present syntheisising the secondary cell wall cellulose, but with the active enzymes being CesA4, CesA7 and CesA8. Figure modified from Cosgrove, (2005).*

So far, the terminal complexes of *Arabidopsis* are the most thoroughly investigated among higher plants. In *Arabidopsis*, CesA1, CesA3 together with CesA2, CesA5, CesA6 or possibly CesA9, make up one six unit rosette terminal complex responsible for making primary cell wall cellulose (Arioli *et al.*, 1998; Scheible *et al.*, 2001; Desprez *et al.*, 2002; Desprez *et al.*, 2007; Persson *et al.*, 2007b; Endler & Persson, 2011), and CesA4, CesA7 and CesA8 together form the secondary cell wall synthesising complex (Taylor *et al.*, 2003). The function of CesA9 is still a bit unclear and it has been suggested that it apart from synthesising primary cell wall cellulose has a function in a seed-specific secondary cell wall synthesising complex (Stork *et al.*, 2010).

Some bacterial species and green algae produce cellulose, although the sizes of the microfibrils are diverse in contrast to the microfibrils of land plants which in almost all cases are around 3.5 nm in diameter. This phenomenon is thought to be due to the appearance of the cellulose synthesising complexes which are found to differ between organisms and species; the terminal complexes in charophycean green algae are, e.g., generally linear with three rows of particles, compared to the circular six unit rosette terminal complex found in land plants. The rosette terminal complexes with its characteristic rosette shape is thought to have arisen in a type of charophycean green algae, one of the closest relatives to land plants (Roberts & Roberts, 2004).

2.5.2 Pectins

Pectins belong to a diverse group of cell wall polysaccharides, all containing a high proportion of galacturonic acid. Pectins are major components of the primary cell wall and the middle lamella, but are almost absent in secondary cell walls. Together the pectins form a hydrated network which contributes to several biological traits and processes of the cell wall such as porosity, pH, cell adhesion and cell wall extension. The major pectins are homogalacturonans, substituted galacturonans, and rhamnogalacturonans. The major industrial use for pectins is as gelling agents in food (Doblin *et al.*, 2003; Fry, 2011).

The backbone of homogalacturonans (HGs) is comprised of partly methyl esterified α-D-(1→4)-galactopyranosyluronic acid (Gal*p*A) units. The degree of methyl esterification plays an important role in the ability of HGs to form gels, and polymers with moderate to low methyl esterification are generally referred to as pectic acid. HGs can account for up to 60% of the primary cell wall pectins (Doblin *et al.*, 2003).

Substituted galacturonans are comprised of a backbone of α-D-(1→4)-Gal*p*A residues. Examples of substituted galacturonans are apiogalacturonan, xylogalacturonan and rhamnogalacturonan II (RGII). While apiogalacturonan and xylogalacturonan are named after its most common substitutions (β-D-(1→2)-apifuranosyl (Api*f*) and β-D-(1→3)-xylopyranosyl (Xyl*p*), respectively, RGII is a complex pectin with at least 4 different side chains which contain some unusual sugar residues, of which some are only found as a component of RGII. RGII is known to form a dimer by a borate cross linkage and mutants affected in RGII synthesis display a cell adhesion phenotype (Doblin *et al.*, 2003; Iwai *et al.*, 2002). RGII is highly conserved among higher plants. Studies have shown that the polymer in a similar form, is present in primary cell walls which derive from the aerial portion of lower plants in amounts comparable to angiosperm primary cell walls (<3% cell wall dry weight; Matsunaga *et al.*, 2004). Importantly, the bryophytes are an exception to this generalisation; although RGII is present, the amount is only approximately 1% of the content in angiosperm cell walls. Notably these results origins from experiments done on the areal portion of plants, which for all plants investigated is the sporophyte generation but which in bryophytes is the gametophyte generation.

Rhamnogalacturonans (RGs), also referred to as RGI, contain a repeating α-D-(1→4)-Gal*p*A-α-L-(1→2)-rhamnopyranosyl (Rha*p*) backbone sequence and side chains of varying complexity from only one glycosyl residue to more than 20. The functions of RGs are largely unknown but they seem to play a role in plant development. RGI is unique among pectins in its ability to form hydrogen bonds to cellulose (Doblin *et al.*, 2003).

2.5.3 Hemicelluloses

Hemicelluloses are defined as polysaccharides that are soluble in strong alkali. The major function of hemicelluloses is to form hydrogen bonds to cellulose so that they together can can provide rigidity to the cell wall network. The main groups of hemicelluloses found in plant cell walls are xyloglucans, mannans, xylans and mixed-linked glucans. The distribution and possibly structure of hemicelluloses in the cell wall has been observed to vary depending on tissue, plant species and cell type (Carpita, 1996; Freshour *et al.*, 1996; Peña *et al.*, 2008; Herve *et al.*, 2009). Hemicelluloses are unwanted wood-components in dissolving pulp, which traditionally is composed of highly purified cellulose, and thus negatively affect the paper yield from woody biomass. In less purified paper-grade pulp, the hemicelluloses contribute to a higher yield and have in addition proved to improve the strength properties of the paper (Puls *et al.*, 2006).

Xyloglucans make up 10-20% of the primary cell wall and are the best studied hemicelluloses. They consist of a β-D-(1→4)-Glc*p* backbone which can be substituted with α-D-(1→6)-Xyl*p*-; α-L-(1→2)-fucopyranosyl (Fuc*p*)-β-D-(1→2)-galactopyranosyl (Gal*p*)-α-D-(1→6)-Xyl*p*-; β-D-(1→2)-Gal*p*-α-D-(1→6)-Xyl*p*-; α-L-(1→2)-Arabinofuranosyl (Ara*f*)-α-D-(1→6)-Xyl*p*; and β-L-(1→3)-Ara*f*-α-L-(1→2)-Ara*f*-α-D-(1→6)-Xyl*p* side chains. Some of the side chains are also acetylated. Although the structure of xyloglucan is generally conserved among higher plants with a few exceptions, grass xyloglucan differs from xyloglucan from type II cell walls in that it contains no or very little fucose and in general is less branched (Doblin *et al.*, 2003). Bryophytes have XGs that are substituted with side chains that differ in structure compared to the XG side chains in higher plants (Peña *et al.*, 2008).

Mannans are polysaccharides containing β-D-(1→4)-mannopyranosyl (Man*p*) residues. Glucomannans have a backbone that contains both β-D-(1→4)-Man*p* and β-D-(1→4)-Glc*p*. Galactoglucomannans have a similar backbone but some of the β-D-Man*p* residues are substituted with α-D-Gal*p* and β-D-Gal*p*-(1→2)-α-D-Gal*p* side chains. Galactomannans are substituted with α-D-Gal*p* residues and are found as major seed storage polysaccharides in some plants, such as legume species. Mannans are not very abundant in angiosperm primary cell walls, but are found in a variety of angiosperm organs, including a significant amount in *Arabidopsis* secondary cell walls and are the major hemicelluloses found in the lignified secondary cell walls of gymnosperms (Bochicchio & Reicher, 2003; Handford *et al.*, 2003).

Xylans are comprised of a β-D-(1→4)-Xyl*p* backbone (Fig. 7A), in most cases substituted with α-L-(1→2 or 1→3)-Ara*f*, α-(4-*O*-methyl)-α-D-(1→2)-glucopyranosyluronic acid (α-D-MeGlc*p*A) and unmethylated α-D-(1→2)-

Glc*p*A side-branches which can also be in the acetylated form (Fig. 7A; Aspinall, 1980; Ebringerova & Heinze, 2000; Brown *et al.*, 2007; Decou *et al.*, 2009). *Populus*, birch, *Arabidopsis* and spruce all exhibit a complex tetrasaccharide made up of -β-D-(1→4)-Xyl*p*-(1→3)-α-L-Rha*p*-α-D-(1→2)-Gal*p*A-β-D-(1→4)-Xyl*p* (Johansson & Samuelson, 1977; Andersson *et al.*, 1983; Peña *et al.*, 2007; Lee *et al.*, 2009b) at the reducing end of the xylan polysaccharide (Fig. 7B). This tetrasaccharide has been reported to be absent in xylan isolated from cell walls of grasses and in the bryophyte *Physcomitrella* (Fincher, 2009; Kulkarni *et al.*, 2012).

Xylans are named after their type of substitutions, which together with their degree of acetylation affects the solubility of the xylan polymer, and so affects the properties of the cell wall. Arabinoxylans (AXs) is predominantly found in the endosperm cell walls in cereals and glucoarabinoxylans (GAXs) are the dominant forms of hemicelluloses in grass type II primary cell walls. GAX is the dominant form of xylan in gymnosperm secondary cell walls, where it constitutes a minor amount of the total hemicellulose content (Bochicchio & Reicher, 2003). GAXs is present also in the dicot type I primary cell wall where it makes up about 5% of the total dry weight content (Darvill *et al.*, 1980; Zablackis *et al.*, 1995; Herve *et al.*, 2009). Glucuronoxylans (GXs) is the dominant form of xylan in the angiosperm secondary cell walls. In *Populus*, secondary cell walls can constitute up to 23% of the total cell wall content and as much as 92 % of the total hemicellulose content (Teleman *et al.*, 2000; Decou *et al.*, 2009).

The high degree of similarity in the structure of xylan across a range of different species suggests that there may be conservation of the enzymatic machinery required for its synthesis (Johansson & Samuelson, 1977; Andersson *et al.*, 1983; Peña *et al.*, 2007). So far, most knowledge that concerns xylan biosynthesis has been gained from investigations into GX biosynthesis in dicots, but studies indicate conservation between parts of the xylan biosynthesis machinery between dicots and monocots and so between GX and GAX (Zeng *et al.*, 2010).

In *Arabidopsis*, a number of enzymes from the GT47, GT43 and GT8 GT families have been shown to be involved in xylan biosynthesis (Table 1). Results obtained in I and II in this thesis has significantly contributed to the identification of *At IRREGULAR XYLEM (IRX)10*, *AtIRX9* and *AtIRX14* and a functional ortholog to each of these genes which are thought to be involved in the synthesis of the GX backbone in *Arabidopsis* (Fig. 7A; Brown *et al.*, 2007; Lee *et al.*, 2007a; Peña *et al.*, 2007; Brown *et al.*, 2009; Wu *et al.*, 2009; Keppler & Showalter, 2010; Lee *et al.*, 2010; Wu *et al.*, 2010). The similarity in phenotypes between the double mutants of the homologous pairs, the fact

that they are non-redundant, and that overexpression of any of the proteins give no phenotypes has led to the hypothesis that IRX10, IRX9 and IRX14 form a xylan backbone biosynthesis complex (Peña *et al.*, 2007; Persson *et al.*, 2007a; Brown *et al.*, 2009; Lee *et al.*, 2009c; Wu *et al.*, 2009; Keppler & Showalter, 2010; Lee *et al.*, 2010; Wu *et al.*, 2010) A similar type of complex which involves close homologs to AtIRX10 and AtIRX14 is thought to exist in wheat, synthesising the GAX backbone. In wheat, an additive protein belonging to the GT75 family of GTs (TaGT75-4) is also suggested to take part in such complex but no homolog to this protein has so far been identified as a putative xylan biosynthesis candidate in *Arabidopsis* (Zeng *et al.*, 2010).

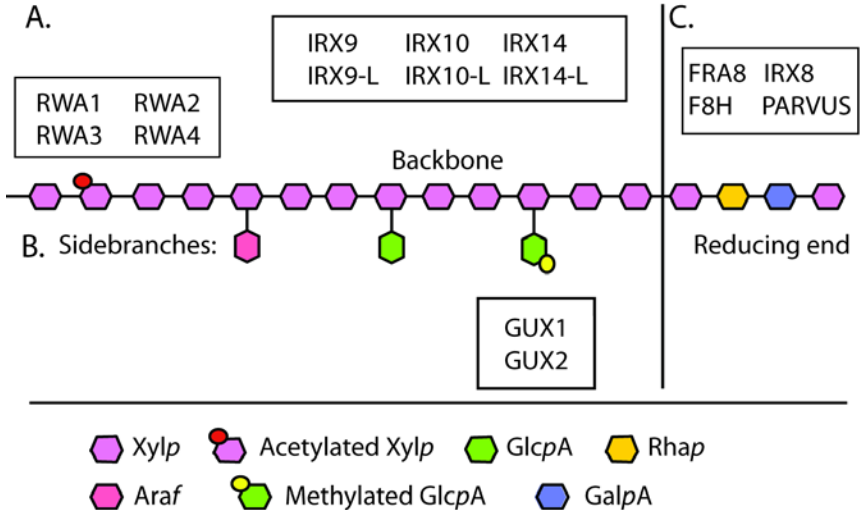

Figure 7. Schematic drawing of the glucoronoxylan backbone (A) with sidebranches (B) and the reducing end oligosaccharide (C). Arabidopsis proteins associated with GX biosynthesis are indicated in boxes close to their putative product.

A further three genes, namely *AtFRA8*, *AtPARVUS* and *AtIRX8*, have been associated with xylan biosynthesis in arabidopsis and are thought to function in the synthesis of the reducing end tetrasaccharide (Fig. 7C; Brown *et al.*, 2007; Peña *et al.*, 2007). FRA8, in common with IRX10, IRX9 and IRX14 also has a functional ortholog in arabidopsis (Zhong *et al.*, 2005; Lee *et al.*, 2007b; Peña *et al.*, 2007; Persson *et al.*, 2007a; Lee *et al.*, 2009a; Wu *et al.*, 2010). Two GTs, At GLUCURONIC ACID SUBSTITUTION OF XYLAN (GUX)1 and GUX2, have been identified to function in the addition of the 4-*O*-Me-GlcUA side-branches to the GX backbone (Fig. 7B; Mortimer *et al.*, 2010) and four closely related ATs called At REDUCED WALL ACETYLATION 1 (RWA1),

RWA2, RWA3 and RWA4 have been identified to function in the acetylation of secondary cell wall xylan (Lee *et al.*, 2011a).

Table 1. Arabidopsis genes associated with GX biosynthesis.

Gene name	Gene number	Enzyme family	Activity	Reference
IRX10	At1g27440	GT47	Xylan backbone	(Brown *et al.*, 2009; Wu *et al.*, 2009)
IRX10-L	At5g61840	GT47	Xylan backbone	(Brown *et al.*, 2009; Wu *et al.*, 2009)
FRA8	At2g28110	GT47	Xylan reducing end	(Brown *et al.*, 2007; Peña *et al.*, 2007)
F8H	At5g22940	GT47	Xylan reducing end	(Lee *et al.*, 2009c; Wu *et al.*, 2010)
IRX9	At2g37090	GT43	Xylan backbone	(Brown *et al.*, 2007; Lee *et al.*, 2007a; Peña *et al.*, 2007; Wu *et al.*, 2010)
IRX9-L	At1g27600	GT43	Xylan backbone	(Lee *et al.*, 2010; Wu *et al.*, 2010)
IRX14	At4g36890	GT43	Xylan backbone	(Brown *et al.*, 2007)
IRX14-L	At5g67230	GT43	Xylan backbone	(Keppler & Showalter, 2010; Lee *et al.*, 2010; Wu *et al.*, 2010)
GUX 1	At3g18660	GT8	Adding glucuronic side branches to the xylan backbone	(Mortimer *et al.*, 2010)
GUX 2	At4g33330	GT8	Adding glucuronic side branches to the xylan backbone	(Mortimer *et al.*, 2010)
IRX8	At5g54690	GT8	Xylan reducing end	(Brown *et al.*, 2007; Peña *et al.*, 2007)
PARVUS	At1g19300	GT8	Xylan reducing end	(Brown *et al.*, 2007; Lee *et al.*, 2007b)
RWA1	At5g46340	AT	Acetylation of the xylan backbone	(Lee *et al.*, 2011a)
RWA2	At3g06550	AT	Acetylation of the xylan backbone	(Lee *et al.*, 2011a)
RWA3	At2g34410	AT	Acetylation of the xylan backbone	(Lee *et al.*, 2011a)
RWA4	At1g29890	AT	Acetylation of the xylan backbone	(Lee *et al.*, 2011a)

Xylans and mannans are found as the major cell wall polysaccharides in some siphoneous green algae, but importantly the xylan in algae is comprised of a (1→3)-linked xylan backbone in contrast to the (1→4)-linked xylan found in angiosperm cell walls (Frei & Preston, 1964). Presence of (1→4)-linked GX has recently been confirmed in the bryophyte *Physcomitrella* (Moller *et al.*, 2007; Kulkarni *et al.*, 2012). Presence of (1→4)-linked xylan in bryophytes is interesting as it suggests that (1→4)-linked xylan is specific not only for higher

plants, but for land plants in general. The *Physcomitrella* xylan differ from GX in higher plants in that it in addition to the (1→4) linked Xylp backbone also displays (1→2→4) linked Xyl*p* residues, that it lack the α-D-MeGlc*p*A side chain and that it (like grass GAX) is reported to lack the reducing end tetrasaccharide.

2.5.4 Phenolic compounds

Lignins are complex polymers built up of monolignols (hydroxyphenyl (H) guaiacyl (G) and syringyl (S) units), joint together by radical coupling. The chemical composition and 3D structure of the lignin polymers are not completely identified due to its complexity and the lack of lignin specific non-destructive extraction methods. The type and proportion of monolignols present in lignin vary depending on species, the age of the plant, and the specific growth conditions.

Lignins provide rigidity to the plant body, and waterproof the water conducting cells in the vasculature of the plant (Sarkanen & Ludwig, 1971). Polysaccharides and lignins are thought to be bound covalently and non-covalently to form lignin-carbohydrate complexes (LCCs). One such interaction suggested by experiments are covalent linkages between lignin and heteroxylans (Lapierre *et al.*, 2001).

Lignification of the secondary xylem cells starts at the cell corners in the middle lamella and then progresses inwards, sealing pores, and compacting first in the primary cell and last the innermost secondary cell wall layers. Experiments indicate that although the process of lignification in itself is not enzymatically driven, environmental conditions and non-enzymatic proteins present are involved in an organized initiation of the process (Vallet *et al.*, 1996; Donaldson, 2001).

Lignification is considered of major importance for the adaptation of plants to live on land. Phenolic compounds have previously been reported in lower plants but true lignin has been thought to be restricted to vascular plants. Studies suggest that lignin is present in red algae (Martone *et al.*, 2007), although this is considered to be a result of convergent evolution. Recent studies of charophycean green algae, the closest relative to land plants, has proved it to contain lignin or lignin-like compounds although in a very low amounts (Sørensen *et al.*, 2011). More experiments are needed to elucidate whether the lignin found in charophycean green algae is an example of convergent evolution or if it has the same evolutional origin as the lignin in land plants.

2.5.5 Non-enzymatic cell wall proteins

A number of non-enzymatic cell wall proteins such as expansins, wall-associated kinases (WAKs), glycine-rich proteins (GRPs) and hydroxyprolinerich glycoproteins (HRGPs) comprise up to 10% dry weight of the primary cell wall composition (Johnson *et al.*, 2003; Riese *et al.*, 2003; Kanneganti & Gupta, 2008).

Expansins are a group of proteins involved in the process of cell wall loosening. There are two major types of expansins; α- and β-expansins. α-expansins are hypothesized to make the glycan chains of a microfibril to dissociate from each other and thereby be more susceptible for enzymatic attacks, while the activity of the β-expansins is more debated (Xu *et al.*, 2010).

WAKs are major players in the physical interaction between the cell wall matrix and the plasma membrane. They are up-regulated by wounding and pathogen infection and are thought to use their cytoplasmic kinase domain to signal cellular events (Kanneganti & Gupta, 2008). Some WAKs have been found to be associated with pectic polysaccharides and some with GRPs. Disruption of WAKs has been found to affect cell expansion (Riese *et al.*, 2003; Kanneganti & Gupta, 2008).

HRGPs are a diverse group of proteins including extensins, arabinogalactan proteins (AGPs) and proline rich proteins (PRPs). Together with the GRPs, these proteins are often called structural proteins as they influence and strengthen the mesh-like structure of the cell wall by their ability to interact with the major cell wall polysaccharides (Johnson *et al.*, 2003). The nomenclature for the structural proteins is initially based on the presence of certain amino acids or motifs, but is under modification along with the progress of molecular science. Evidence for covalent cross-linkage to pectins has been found for some of the proteins and both HRGPs and GRPs interact with lignin and play a possible non-catalytic role in the initiation of the lignification process (Johnson *et al.*, 2003).

3 Objectives

The main objective of the work done in this thesis was to elucidate the involvement of *IRREGULAR XYLEM 10 (IRX10)* gene family members in biosynthesis of glucuronoxylan, a major component of the angiosperm secondary cell wall.

The main objective of paper I was to assign a function to the *Arabidopsis* IRX10 and IRX10-L proteins, and in paper II the main objective was to figure out the relationship between the *Arabidopsis IRX10* genes and three other gene families (*IRX14*, *IRX9* and *FRA8*) based on the high similarity in expression pattern between members of the four gene families.

In paper III, the role of *Populus IRX10* related genes were investigated, again based on the expression pattern which in both *Arabidopsis* and *Populus* is specific for secondary cell wall producing tissues. The main objective was to find out if the functions of the *IRX10* related genes are functionally conserved between *Populus* and *Arabidopsis*.

Paper IV is based on the high sequence similarity between the PpGT47D protein of *Physcomitrella* and the IRX10 related proteins of *Arabidopsis* and *Populus*. The main objective was to further investigate the degree of functional conservation of the IRX10 related protein and its putative role in GX biosynthesis in *Physcomitrella*.

4 Methodological Overview

4.1 Model Systems

There are at least 300 000 plant species on earth. The plant research community has chosen to focus on just a few plants and develop good systems for in depth studies, with the assumption that much of the knowledge that is gained is applicable to other species. Once a biological pathway or pattern is established in one species, it can be used to show similarity among several species or that it is unique for a certain species. There are a number of features that make a model species particularly suited for research purposes including a rapid lifecycle, that it readily reproduces, they are easy to maintain and ideally take up little growth space. In addition it is important for molecular based approaches that the model organism has a genome of manageable size which preferably is sequenced and is possible to transform. Once these demands are fulfilled the important thing is that the plant also displays the trait that is the focus for the study. Work in this thesis has utilised the woody trees *Populus trichocarpa* and *Populus tremula* x *tremuloides* (the genera referred to as *Populus*), the herbaceous *Arabidopsis thaliana* (referred to as *Arabidopsis*) and the bryophyte *Physcomitrella patens* (referred to as *Physcomitrella*); with *Populus* and *Arabidopsis* being well established model organisms belonging to the group of higher plants and *Physcomitrella* belonging to the group of lower plants. So far, most information regarding the biosynthesis of the cell wall has been gained in *Arabidopsis*, but during recent years knowledge about the economically more important cell wall of *Populus* plants has started to emerge. Cell wall biosynthesis in bryophytes is largely unknown. By combining work in the three organisms, I have been able to continue the identification of GTs important for the cell wall biosynthesis in *Arabidopsis* and *Populus*. In addition, the work done in *Physcomitrella* has initiated the unraveling of differences regarding xylan biosynthesis which might have had major evolutionary consequences during the development of higher and lower plants.

4.1.1 Arabidopsis

Arabidopsis is considered a weed and the plant has no economic importance, but due to the long tradition of using *Arabidopsis* within plant research, it is considered a valuable model organism. Consequently, molecular methods are well developed, *Arabidopsis* mutants are readily available, and many molecular pathways have been identified. The genome was sequenced in year 2000 and is composed of 125 megabase-pairs (Mbp) distributed across 5 chromosomes (Kaul *et al.*, 2000). With a final size of approximately 40 cm in height, it is easy to handle as it can be grown in numerous numbers within a compact space in controlled climate chambers. *Arabidopsis* is an annual herbaceous species. It has both male and female reproductive organs on the same plant and goes through one life cycle from seed to seed in about 6-8 weeks.

As in all vascular plants, the xylem cells of the *Arabidopsis* vasculature are strengthened by secondary cell walls. Furthermore, the vasculature is rigidified by secondary growth, in which the cambium gives rise to several rows of strengthened and lignified xylem cells, forming the secondary xylem (Fig. 8). In *Arabidopsis*, the formation of secondary xylem is pronounced in specific organs and under certain conditions. The tissue formed in the hypocotyls grown under short day conditions (8h light/16h dark) has for example been suggested to resemble the secondary xylem of trees (Chaffey *et al.*, 2002; Nieminen *et al.*, 2004; Zhang *et al.*, 2011). This implies that all components necessary for secondary cell wall biosynthesis and the formation of a secondary xylem are present in *Arabidopsis* and suggests it to be a model system that is well suited for studies into the secondary cell wall.

4.1.2 Populus

The *P. trichocarpa* genome was sequenced in 2006, and contains approximately 480 Mbp distributed across 19 chromosomes (Tuskan *et al.*, 2006). Hybrid aspen can be transformed via *Agrobacterium* mediated approaches, and the most commonly used line for this process is called T89 (Nilsson *et al.*, 1992). The growth rate of *Populus* is relatively fast for a woody species. Despite the relatively large size in comparison to *Arabidopsis* (Fig. 9), young *Populus* plants are small enough to be grown in greenhouse or controlled climate chambers. As *Arabidopsis* it can reproduce sexually although *Populus* plants in contrast to *Arabidopsis* are dioecious. Being perennial species, *Populus* normally set seed for the first time after around ten to fifteen years, and can stay alive hundreds of years under the right conditions. Despite the relatively old age at which they produce seed, *Populus* plants can easily be clonally propagated which make them suitable for laboratory research.

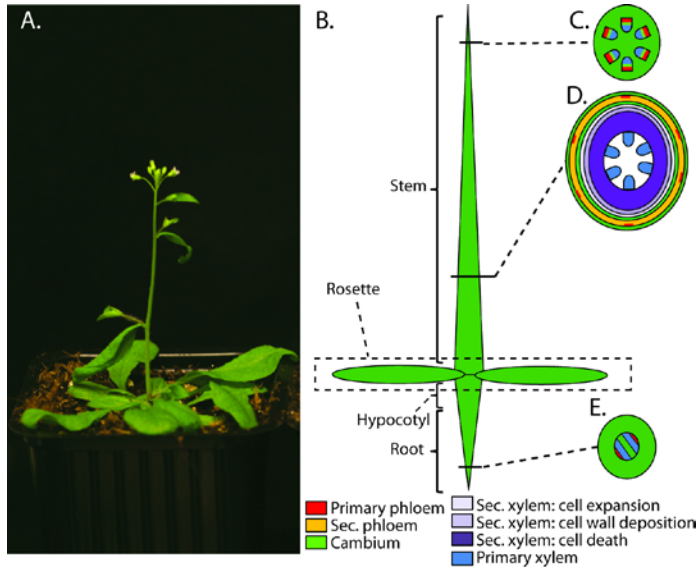

Figure 8. The Arabidopsis stem goes through primary and secondary growth, and produces primary and secondary cell walls. (A) A wild-type Arabidopsis plant. (B) Schematic picture of an Arabidopsis plant. (C) The primary xylem is viewed as a cross-section with the vascular bundles visible. (D) The stem (E) and the root (F) viewed as cross-sections with the vascular tissues indicated. Modified from Nieminen et al.(2004).

Figure 9. A one months old T89 hybrid aspen with a young Arabidopsis *wild-type plant slotted in to the right showing approximate size differences between the two species.*

Being woody species the secondary xylem dominate the *Populus* plants. I have taken advantage of similarities of the secondary xylem between *Arabidopsis* and the woody *Populus trichocarpa* (*Pt*; black cottonwood), and the *Populus tremula* x *tremuloides* hybrid (*Ptt*; common aspen x quaking aspen); in this thesis referred to as hybrid aspen (Chaffey *et al.*, 2002; Nieminen *et al.*, 2004; Ubeda-Tomas *et al.*, 2007). The anatomical organisation and the components making up the cell walls of *Populus* plants are similar to the *Arabidopsis* cell wall, but one important difference between the secondary growth of *Arabidopsis* and *Populus* is the presence of ray parenchyma cells in the xylem of *Populus* (Chaffey *et al.*, 2002; Nieminen *et al.*, 2004). Comparative expression studies have suggested that many protein sequences of GTs involved in cell wall biosynthesis are conserved between *Arabidopsis* and *Populus* (Hertzberg *et al.*, 2001a; Aspeborg *et al.*, 2005; Ubeda-Tomas *et al.*, 2007; Yang *et al.*, 2011), and in depth studies have confirmed functional conservation of *Arabidopsis* xylan biosynthesis enzymes in *Populus* (Zhou *et al.*, 2006; Zhou *et al.*, 2007; Kong *et al.*, 2009; Lee *et al.*, 2009a; Lee *et al.*, 2009b; Lee *et al.*, 2011b; Li *et al.*, 2011).

4.1.3 Physcomitrella

To bring in a non-vascular species and thereby an evolutionary aspect to this study, the bryophyte *Physcomitrella patens* was used as a model system. The sequencing of the *Physcomitrella* genome was completed in year 2008 with a size of the genome estimated to approximately 480 Mbp distributed across 27 chromosomes. The availability of genomic sequences in combination with the ability to use homologous recombination for introduction or depletion of genes (see chapter 4.3) has made it a suitable model organism for genetic studies (Rensing *et al.*, 2008). In addition *Physcomitrella* is easy to maintain under laboratory conditions since it takes very little space and is easy to propagate as any part of the organism can be used to initiate further growth or to produce clones.

The dominant life form of higher plants such as *Arabidopsis* and *Populus* is the diploid generation. In contrast, the dominant stage of the bryophyte lifecycle is the haploid gametophytic generation, and only the setae and the calyptra forms a diploid sporophyte (Fig. 10). Sexually, *Physcomitrella* is a monoecious plant, with both archegonia and antheridia being produced on the same gametophore.

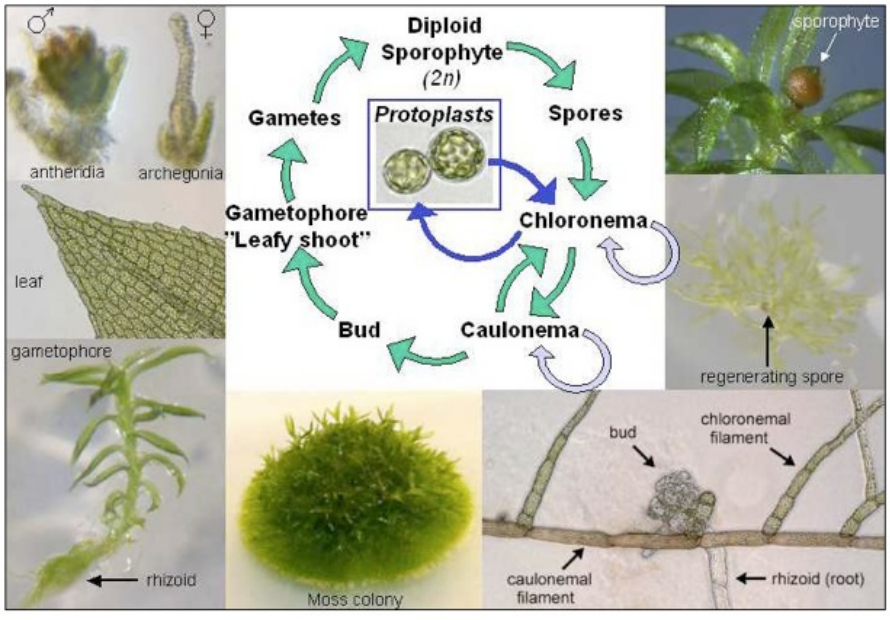

Figure 10. The lifecycle of Physcomitrella patens. Figure credit Centre for Functional Genetics, Uppsala University (www.cfg.uu.se)

Although distantly related, *Physcomitrella* has proven to share many basic molecular pathways with higher plants such as the auxin signaling pathway, regulation of the cytoskeleton for tip growth, mechanisms important for filamentous growth/root hair development and ABA-mediated desiccation tolerance (Prigge & Bezanilla, 2010).

Physcomitrella belongs to the group of non-vascular plants. Despite this, it contains specialised conducting strands made of thin-walled non-lignified cells called hydroids. The water conducting cells are found in the sporophytic seta, the gametophore stem, and in the midrib of the leaves (Ligrone *et al.*, 2000; Sakakibara *et al.*, 2003). Although not found in *Physcomitrella*, mosses that belong to the *Polytrichopsida* have cells with thickened cell walls making up the mid strands of the gametophyte stem. The mid strand in *Polytrichopsida* also contains cells called leptoids that are specialised in nutrient transport. In other mosses, leptoids occur widely in immature sporophytic setae, but are not present in the gametophytic generation, where the nutrients most probably are transported by the aid of less specialised elongated parenchyma cells situated in the cortical tissue of the leafy stem (Ligrone *et al.*, 2000).

Physcomitrella has no secondary growth, and does not produce secondary cell walls comparable with secondary cell walls in higher plants. Despite this, almost all components making up the cell walls of higher plants (including

(1→4)-linked xylan) have been detected in *Physcomitrella* cell walls (Moller *et al.*, 2007; Kulkarni *et al.*, 2012). An exception to this is lignin which is not found in *Physcomitrella* (Ligrone *et al.*, 2000). The *Physcomitrella* cell wall is interesting to investigate further from an evolutionary perspective, as the presence of highly conserved cell wall GTs indicates that some of the machinery for making the components of the angiosperm cell wall were already present in a common ancestor to the two groups (Kulkarni *et al.*, 2012). Furthermore, the exact composition of the cell walls in sporophytic seta cells is not well investigated due to the difficulty of obtaining sufficient material for analysis. I have chosen to use *Physcomitrella* in my studies due to the finding that the *Physcomitrella* genome contains a gene encoding a highly conserved homolog to the *Arabidopsis* IRX10 protein, which is thought to be involved in secondary cell wall GX biosynthesis (I). As *Physcomitrella* cells lack secondary cell walls, the plant clearly has potential to give new information about the function and evolution of the *IRX10* gene family.

4.2 Downregulation of Genes

One way to obtain information about the function of a gene is to use molecular methods to either down-regulate or knockout the expression of the gene in plants, and to phenotypically investigate the resulting plants. The down-regulation can be done in several ways, and depending on the method used can either cause a complete loss of transcript or a down regulation (i.e. partial loss). The resulting lines are called knock-outs or knock-downs. To obtain a complete knock-out, it is necessary that the disruption of the gene is introduced at the gene specific site in the genome. Furthermore, the disruption has to be such that the function of the protein cannot be performed by partial transcription of the gene.

I have in this thesis used two types of knock-out mutants; transfer-DNA (T-DNA) insertion lines in *Arabidopsis* (Fig. 11A) and lines with genes disrupted by homologous recombination in *Physcomitrella* (Fig. 11B). T-DNA lines are produced by screening numerous lines transformed with T-DNA cassettes to find those disrupted in the gene of interest (Alonso, 2003). In contrast, homologous recombination relies on homologous sequences in the knock-down construct to direct it to the gene of interest by an endogenous mechanism present in only certain organisms including *Physcomitrella*. To trigger the mechanism, the construct for disruption is introduced in excess via polyethylene glycol (PEG) treatment of protoplasts (Schaefer *et al.*, 1991).

To obtain a knock-down the most common methods used are antisense, RNA interference (RNAi) and more recently micro-RNAi (miRNAi). In these

methods, the knock-down of a gene is caused by the introduction of a gene specific antisense sequence into the genome, which when expressed in the form of small interfering RNA (siRNA) binds to matching messenger RNA (mRNA) sense sequences and triggers mechanisms that leads to the destruction of transcripts (Fig. 11C). The introduction of the knock-down sequences are in most cases made by *agrobacterium* mediated transformation (Clough & Bent, 1998), which randomly inserts the knock-down construct into the genomic sequence of the host species. Knock-downs can be achieved in *Arabidopsis* and *Physcomitrella* in addition to the previously mentioned knock-out methods, but is the only way at present to obtain down regulation of genes in *Populus* species (Nilsson *et al.*, 1992).

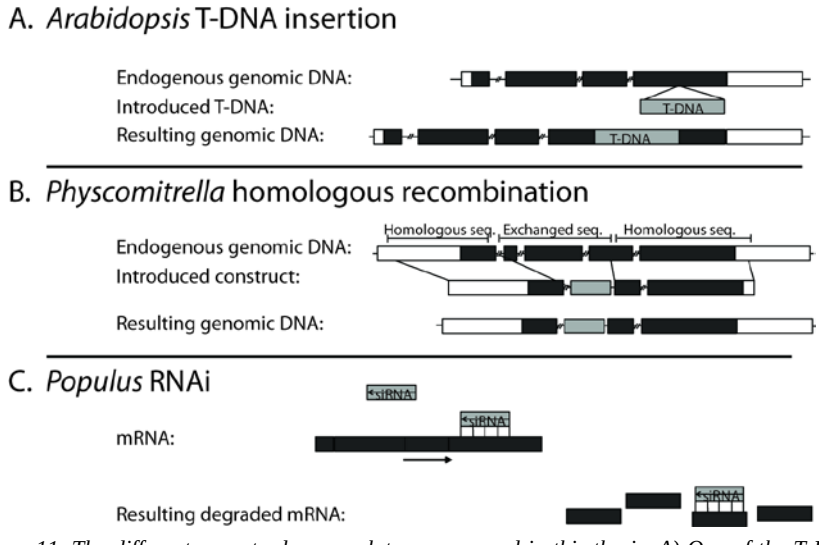

Figure 11. The different ways to downregulate a gene used in this thesis. A) One of the T-DNA insertions used to knock-out the AtIRX10 gene family members in Arabidopsis. *B) The knock-out construct used to down regulate PpGT47Din* Physcomitrella. *C) The RNAi technique used to down regulate the IRX10 gene family in* Populus. *Arrows indicates the direction of the DNA 5' to 3'.*

4.3 Functional Assays

In order to elucidate the function of a GT, one way is to use *in vitro* systems of which the most common is to heterologously express the protein in a host organism. The host secretes the enzyme into the surrounding media which is provided with potential substrates and acceptors, and any synthesized product is subsequently measured. This type of enzymatic assay has been tested for several of the presumed GX biosynthesis enzymes but with no success

(Winzell *et al.*, 2010). The negative results together with other experiments (Lee *et al.*, 2012) have led to the interpretation that the GX biosynthesis enzymes in their active forms are bound in a complex. This implies that the other protein partners in a putative complex also have to be present in correct amounts for the enzymatic activity to work properly (Winzell *et al.*, 2010). In addition some of the plant GTs are known to be promiscuous and can have several endogenous functions, or alternatively can be forced to use other substrates similar to the true one if the provided substrate is presented in excess and if no competing substrates are present. GTs can be specific to one substrate or one acceptor when expressed in the plant, but does not necessarily have to be specific in a slightly different environment or in an *in vitro* expression system.

In order to overcome the problem of missing interaction partners, an *in vitro* assay system utilising microsomes, plant derived membrane fractions, has been used. In this assay, knock-out mutants lacking the enzymes of interest are used. Microsomes are extracted from the knock-out mutants and fed with substrate and acceptors. The synthesised products are compared with products produced by microsomes extracted from wild-type plants. If no products can be found it indicates that the plant is unable to synthesise the product without the presence of the enzyme of interest. This has successfully been done with several knock-out plants that are thought to have impaired GX biosynthesis, and has contributed to the conclusion that a number of GTs are considered strong candidates for GX biosynthesis (Brown *et al.*, 2007; Brown *et al.*, 2009). This type of *in vitro* assay is clearly informative but is importantly not providing direct evidence of enzymatic function. Just before printing this thesis, a microsome experiment performed on tobacco tissues with heterologously expressed *Arabidopsis* IRX9 and IRX14 proteins, was published (Lee *et al.*, 2012). In this experiment the microsomes were fed UDP-Xyl substrate and Xylp_n acceptors. Results prove that IRX9 and IRX14 work together and are involved in GX backbone elongation, and clearly suggest them to be xylosyltransferases (XylT). The exact enzymatic mechanisms and the nature of the interaction between the two enzymes, is however still to be elucidated.

A third way to try to investigate the enzymatic function of a protein is the type of complementation experiments used in this thesis. In this assay, the gene of interest is expressed in the corresponding knock-out mutant in order to see if the expression can complement the mutant and restore a wild-type appearing plant. In this way, all presumed interaction partners are expected be present in the right amount and in the right environment together with the right amount of substrate and acceptor. This can be done to confirm functional redundancy of two genes (I and II). Complementation experiments are also valuable tools to

confirm functional orthologs as it is possible to transfer homologs across species (III and IV; Zhou *et al.*, 2006; Lee *et al.*, 2009a). However, a protein might have retained its enzymatic function during the evolution it might have evolved together with its interaction partners so that the interaction partners from two species are no longer interchangeable. An ortholog could then display inability to complement a knock-out mutant from another species despite having an equivalent enzymatic activity. Similarly, if two very similar homologs are expressed in the same environment they can sometimes perform the same enzymatic activity due to possessing similar 3D structure and substrate and acceptor binding properties. If proteins in their endogenous environments have developed sub- or neofunctions they can still display the ability to complement a knock-out mutant from another species although it is not the primary endogenous function.

Taken together, for functional assays to be informative it is necessary to know which phenotypic effects to measure and to be able to distinguish primary from secondary effects. A functional assay alone is never enough evidence for enzymatic activity but needs to be supported by complementary experiments, irrespective whether these are performed *in planta* or *in vitro*.

4.4 Studies of Cell Wall Mutants

A thorough investigation of genetically altered plants in comparison to wild-type plants is one way to confirm or support results obtained from functional assays. Phenotypes of a mutant can be obvious, with the plants displaying severe dwarfism or altered spatial or temporal developmental patterns. However, phenotypes can also be more subtle resulting in differences which do not manifest as a visible morphological difference to the wild-type. When investigating cell wall phenotypes, additional chemical analytical methods are often valuable and necessary as the phenotypes are difficult to interpret unless the exact cell wall composition or relative ratio of cell wall components are determined. This thesis has utilised a range of different approaches including three types of chemical analysis; quantitative and qualitative approaches as well as fingerprinting methods.

In the quantitative methods, derivatisation of cell wall polysaccharides was used to determine the monosaccharide amount and composition of the cell wall material. Qualitative methods include immunolocalisation where the presence of certain cell wall polysaccharide epitopes are investigated, and enzymatic digestions where the presence of certain oligosaccharides are looked at in mutants of interest compared to wild-type plants. Finally, Fourier transform infrared spectroscopy (FT-IR) and analytical pyrolysis are termed

fingerprinting methods as the results are based on differences that are not always annotated or traced to certain cell wall components between wild-type and mutants.

4.4.1 Quantitative methods

One basic parameter for chemical characterisation of cell wall mutants is to look at the monosaccharide composition of starch treated alcohol insoluble residues (AIR) prepared from cell walls of the plants. This can be done by quantifying the alditol acetates (AA) derived from the samples as in I and II, or by quantifying glycosides methylated by trimethylsilyl (TMS) as in III and IV. Both AA analysis and TMS analysis are based on the complete hydrolysis of the glycosidic bonds of the cell wall polysaccharides in the sample.

The outcome from the AA analysis is a trace with one peak representing each monosaccharide that can then be compared with known standards. Spiking the sample with a known amount of inositol makes it possible to quantify the amount of each monosaccharide from the area under each peak. Normalization makes it possible to compare samples from different plants. One disadvantage with this method is that the uronic acids (GalA and GlcA) need to be analyzed by additive methods as they do not give peaks in the trace. The outcome from TMS analysis is more complex as the result is a trace where each monosaccharide is represented by four peaks; one for each chemical configuration (α-pyranose, α-furanose, β-pyranose and β-furanose). The amount of monosaccharides is quantified in the same way as for the AA, but importantly this method is also applicable for the uronic acids.

Once the monosaccharide composition has been determined it is possible to use the information to make qualified assumptions about which polysaccharide may be altered either in amount or composition. To further understand and confirm the presumed cell wall alteration it is also necessary to carry out a more qualitative analysis.

4.4.2 Qualitative methods

Immunolocalisation is a commonly used method where antibodies raised against specific polysaccharides are used to label the components of the cell wall samples. Differences in labeling pattern or intensity can then be used to identify differences between species or wild-type and mutants (Moller *et al.*, 2007). One disadvantage with this method is that it is difficult to know exactly which epitopes the antibodies recognize and the antibodies can also be relatively non-specific, binding to other components than the assumed one. Epitopes can further be masked by other cell wall components and a negative result is thus not enough to prove the absence of a specific epitope (Herve *et*

al., 2009). Despite this, differences in labeling patterns between wild-type and mutants are strong indicators of which cell wall components are altered. In this thesis two xylan specific antibodies, LM10 and LM11, have been used (I and II).

In order to find out which cell wall component is affected by the mutation, it is possible to investigate fractions of cell wall derived components. AIR is treated sequentially with an acid (i.e. ammonium oxalate) to obtain a pectin rich fraction, and then a base (i.e. NaOH) for a hemicellulose-rich fraction. Finally, the remaining pellet can be used for cellulose analysis. The fraction of interest from the mutant is then analysed and compared to wild-type.

In this thesis the NaOH fraction was hydrolysed with an endo-xylanase (I and II) and the products were analysed by electrospray ionization mass spectroscopy (ESI-MS) to confirm the presence of the xylan side branches (I). Nuclear magnetic resonance (NMR) was used to confirm the presence of an intact reducing end sequence and to estimate the degree of polymerization of the xylan backbone (I). Finally high-pressure size-exclusion chromatography (HP-SEC) was used to measure the degree of polymerization of the xylan backbone (II).

4.4.3 Fingerprinting methods

FT-IR is based on the measurement of the absorbed energy of freeze-dried samples which are mixed with KBr and irradiated with infrared light. By combining the microscopical measurements with multivariate and normalization analysis software, fingerprint spectrums can be achieved from different samples, tissues or even types of cells. Chemical differences between samples can then be detected. Some of the differences in spectrums can be annotated to differences in abundance or distribution of certain cell wall components (Gorzsas *et al.*, 2011). This method was used in III to investigate the chemotype of *Populus* RNAi lines.

In analytical pyrolysis the sample cell wall material is thermochemically decomposed under exclusion of oxygen. The pyrolytic degradation products. are then separated and detected with gas-chromatography mass spectroscopy (Py-GC/MS; Gerber *et al.*, 2012). The chromatograms from the corresponding samples are then compared in order to detect, classify and evaluate chemical phenotypes. Some of the differences can be annotated to specific components or groups of components that are most likely to be different between the samples. This method is readily used to explore differences in lignin content or composition. In this thesis Py-GC/MS was used in III to further explore the chemotype of *Populus* RNAi lines.

5 Results and Discussion

5.1 GX synthesis in *Arabidopsis* (Paper I and Paper II)

5.1.1 Investigating the function of *Arabidopsis* IRX10 and IRX10-like (Paper I)

A number of genes involved in secondary cell wall biosynthesis have been identified by the secondary cell wall specific expression pattern of *Arabidopsis* and *Populus* homologs (Ubeda-Tomas *et al.*, 2007). One of the genes identified was the *Arabidopsis IRX10*. Its closest tobacco homolog, *NpGUT1*, has previously been suggested to be involved in RGII biosynthesis, functioning to add GlcA to one of the side chains of the polymer (Iwai *et al.*, 2002). In Paper I, the secondary cell wall specific function of *Arabidopsis* IRX10 and its close homolog IRX10-L was investigated further. Double *irx10/irx10-L* mutants were made by crossing T-DNA insertion lines and phenotypes of obtained homozygous and heterozygous lines were evaluated.

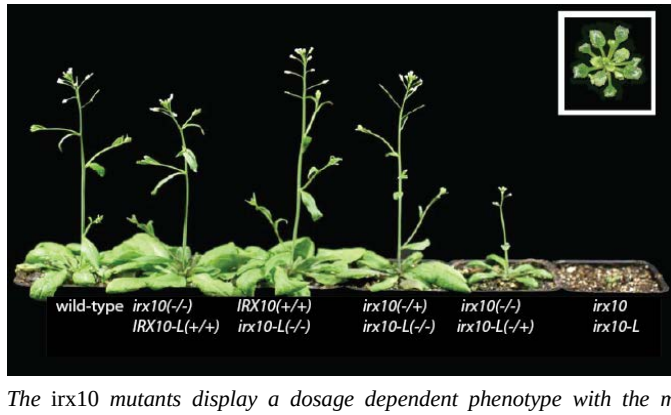

Figure 12. The irx10 *mutants display a dosage dependent phenotype with the most severely affected* irx10/irx10-L *double mutant (higher magnification in the upper right corner of the picture) being severely dwarfed with decreased rosette diameter and smaller leaves (I).*

The *irx10/irx10-L* double mutant and the *irx10/irx10-L (+/-)* plants were severely dwarfed, with decreased rosette diameters and smaller leaf sizes (Fig. 12). Stem sections revealed a strong reduction in secondary cell wall thickness in both the *irx10/irx10-L* double mutants and the *irx10/irx10-L (+/-)* lines, supporting involvement in secondary cell wall formation. The double mutant was most severely affected and could only infrequently form an infertile inflorescence stem if the plants were grown under a protective plastic lid.

The severity of the phenotype was dosage dependent, and the difference in severity between *irx10(+/-)/irx10-L* and *irx10/irx10-L(+/-)* suggested the two proteins have slightly different functional properties. RNAi experiments confirmed dosage dependence for both IRX10 and IRX10-L, and complementation experiments proved functional redundancy as overexpression of both proteins fully restored the double mutant to a wild-type looking appearance. *IRX10* was more efficient in complementing the double mutant if expressed under the *IRX10* native promoter since heterozygous expression fully restored the double mutant to wild-type looking appearance while expression of IRX10-L needed to be homozygous in order to obtain a full rescue. This proves IRX10 to be functionally more efficient in secondary cell wall formation than IRX10-L, and suggests slightly different roles for the two proteins.

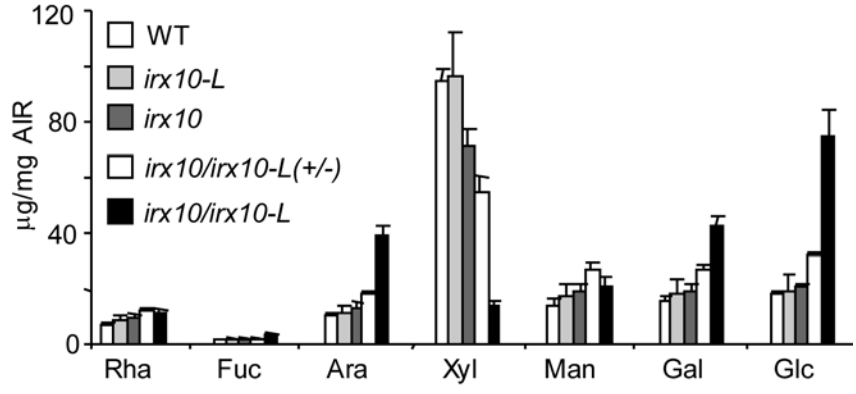

Figure 13. The Atirx10 *mutants display a decreasing amount of xylose compared to wild-type depending on genotype from no reduction in xylose in the* irx10-L *to a strong reduction in the* Atirx10irx10-L *double mutant (I).*

No phenotype with defective cell adhesion could be seen for any of the *Arabidopsis irx10* or *irx10-L* mutants that would indicate involvement in RGII synthesis, but a genevestigator expression analysis (www.genevestigator.com)

suggested strong co-regulation with known GX biosynthesis genes (II). Analysis of the *irx10/irx10-L* double mutant revealed a strong reduction in xylose (Fig. 13) and likewise a strong reduction in signal from xylan specific LM10 and LM11 antibodies in the *irx10/irx10-L* plants, which confirmed involvement of the IRX10 and IRX10-L proteins in xylan biosynthesis. Further, the *irx10/irx10-L(+/-)* plants had reduced xylan backbone length and the tetrasaccharide situated at the reducing end of the xylan polysaccharide was found to be intact in a comparable relative amount to the wild-type in the *irx10/irx10-L(+/-)* plants. Taken together, these experiments provide strong evidence for the IRX10 protein to be involved in GX backbone synthesis.

5.1.2 Investigation of the redundant homologs for IRX9, IRX14 and FRA8, three genes involved in *Arabidopsis* GX biosynthesis (Paper II)

IRX10, IRX9 and IRX14 have been proven to be involved in GX backbone synthesis (Brown *et al.*, 2007; Peña *et al.*, 2007; Brown *et al.*, 2009; Wu *et al.*, 2009) and FRA8, IRX8 and PARVUS have been shown to be involved in the synthesis of the tetrasaccharide situated at the reducing end of the GX polymer (Lee *et al.*, 2007b; Peña *et al.*, 2007; Persson *et al.*, 2007a). The discovery of functionally redundant homologs to IRX10 and FRA8 (Lee *et al.*, 2009c; Wu *et al.*, 2009) has contributed to further understanding of the complexity of the GX biosynthesis pathway. In II, homologs to *IRX9* (*IRX9-L*) and *IRX14* (*IRX14-L*) were identified and investigated further. Double and single mutants were obtained for *IRX9/IRX9-L*, *IRX14/IRX14-L* and *FRA8/F8H*, and the resulting plants were analysed.

The *irx9/irx9-L* and *irx14/irx14-L* and *fra8/f8h* double mutants all exhibited a severely dwarfed dark green phenotype with reduced rosette size and leaves similar to the phenotypes previously described for *irx10/irx10-L* double mutants (Fig. 12; Brown *et al.*, 2009; Lee *et al.*, 2009c; Wu *et al.*, 2009). In addition, the *irx9/irx9-L(+/-)*, *irx14/irx14-L(+/-)* and *fra8/f8h(+/-)* all exhibited an intermediate phenotype as previously described for the *irx10/irx10-L(+/-)*. Furthermore, the thickness of the secondary cell walls were heavily reduced in the double mutants as previously described for the *irx10/irx10-L* double mutant. The cell wall thickness was also reduced compared to wild-type in the *irx9/irx9-L(+/-)*, the *irx14/irx14-L(+/-)* and the *fra8/f8h(+/-)* plants, although the walls were slightly thicker compared to the double mutants. A similar finding was previously described for the *irx10/irx10-L(+/-)* plants.

Complementation experiments showed functional conservation within the homologous pairs (i.e. IRX9 and IRX9-L, IRX14 and IRX14-L, and FRA8 and F8H) but no functional overlap between the genes from different pairs. *AtIRX10* was also unable to complement for the loss of the *fra8/f8h* genes,

although *IRX10* and *FRA8* and their respective homologs share some sequence homology and belong to the GT47 family of glycosyltransferases.

Cell wall analysis of the *irx9/irx9-L*, *irx14/irx14-L* and *fra8/f8h* single and double mutants confirmed a dosage dependent reduction of xylose depending on whether the plants were homozygous or heterozygous. Labelling with LM10 antibodies similarly displayed a dosage dependent reduction of signal from the xylan specific epitope depending on genotype of the plants. The xylan signal was almost completely abolished in the *irx14/irx14-L* and the *fra8/f8h* double mutants as previously shown for the *irx10/irx10-L* double mutant. In contrast, some xylan could still be detected in the *irx9/irx9-L* double mutants. The strongest reduction among the intermediate plants could be seen in the *fra8/f8h* plants. GX analysis performed on the 4 M NaOH soluble fraction showed a strong reduction in GX content in *irx9/irx9-L* and *fra8/f8h* while the *irx14/irx14-L* double mutant, as previously shown for *irx10/irx10-L* double mutant, displayed a complete lack of GX. The degree of polymerisation of the xylan backbone was reduced in all three double mutants (*irx9/irx9-L*, *irx14/irx14-L* and *fra8/f8h*), suggesting an involvement of all these genes in the synthesis of the xylan backbone.

In conclusion, two more proteins involved in *Arabidopsis* GX biosynthesis were identified, namely IRX9-L and IRX14-L. Furthermore, all three homologous pairs investigated in this study (i.e. IRX9/IRX9-L, IRX14/IRX14-L and FRA8/FRA8-L) influence the length of the xylan backbone, although it is unclear whether this influence is direct or indirect for FRA8/F8H that previously have been shown to be involved mainly in the biosynthesis of the tetrasaccharide situated on the reducing end. The occurrence of redundant gene pairs with very similar phenotypes has led to a hypothesis in which the pairs are divided into a major (*IRX9*, *IRX10* and *IRX14*) and minor (*IRX9-L*, *IRX10-L* and *IRX14-L*) set of genes. The major genes are thought to play the most important role in secondary cell wall GX biosynthesis while the minor set of genes could substitute for the major genes under certain developmental stages or environmental conditions perhaps allowing the plant to modify the GX backbone chain length and/or degree of methylation of the GlcA side chains according to need. A hypothetical explanation for the phenotypes of the *irx10/irx10-L* phenotypes is shown in Fig. 14, where the ability of the IRX10-L to substitute for IRX10 can hold the complex together in a less efficient form in the *irx10/irx10-L*(-/+) plants, but the lack of both proteins in the *irx10/irx10-L* double mutants make the complex disintegrate or remain dysfunctional in the absence of the IRX10 proteins. The basic idea can be applied for the *irx9/irx9-L* and *irx14/irx14-L* mutants as well.

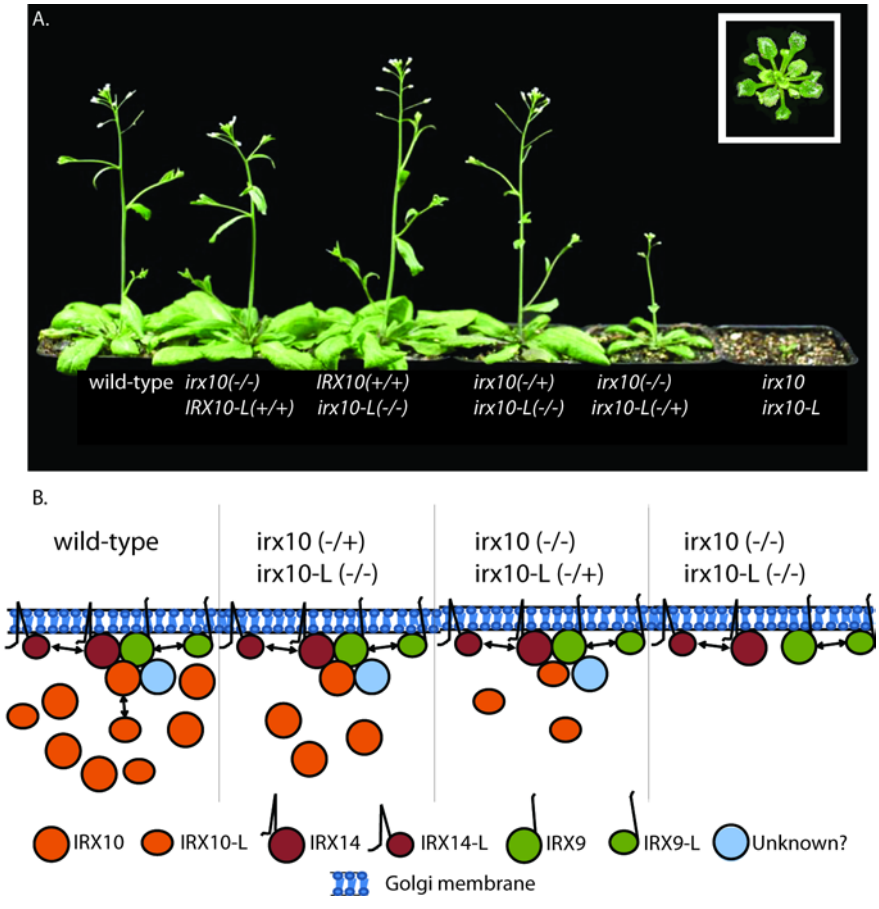

Figure 14. A) A picture of the phenotype of the different irx10 knockout combinations in Arabidopsis. irx9/irx9-L *and* irx14/irx14-L *display the same type of dosage dependent phenotypes (I). B) A hypothetical model of a presumed GX backbone synthesis complex in the Arabidopsis* irx10 *knock-out combinations. The model is applicable also for the* irx9 *and* irx14 *knock-out combinations.*

5.2 The *IRX10* gene family in *Populus* (Paper III)

PttGT47A, a close hybrid aspen homolog to the *Arabidopsis* IRX10 protein, has been identified in a screen performed in order to find candidates for secondary cell wall synthesising enzymes (Hertzberg *et al.*, 2001b; Ubeda-Tomas *et al.*, 2007). In III, the *P. trichocarpa* genome was blasted for close homologs of IRX10. The four full length gene models found (*PtGT47A-1*, *PtGT47A-2*, *PtGT47D-1* and *PtGT47D-4*) were investigated further in hybrid aspen and *Arabidopsis*. The hybrid aspen *PttGT47A* and *D* genes were targeted by RNAi

and the knock-down lines were analysed. Overexpression constructs were made and introduced into T89 wild-type hybrid aspen. Additionally, gene constructs overexpressing the cDNA were used for complementation studies in *Arabidopsis irx10/irx10-L* double mutants.

Although three lines with significant reduction of *PttGT47A* expression and at the same time a moderate reduction of the *PttGT47D* expression were obtained, no cell wall phenotype could be detected in the RNAi lines, and the involvement of the *Populus GT47A* and *D* genes in secondary GX biosynthesis could not be confirmed. *Arabidopsis* complementation studies showed that the *Populus* GT47A-1 and A-2 proteins are functional orthologs of the *Arabidopsis* IRX10, while GT47D-1 and D-4 were only able to partly restore the phenotype of the *Atirx10/irx10-L* double mutant. A plausible explanation for the phenotypes of the complemented plants is shown in Fig. 15, using the same hypothetical complex model as previously in Fig. 14.

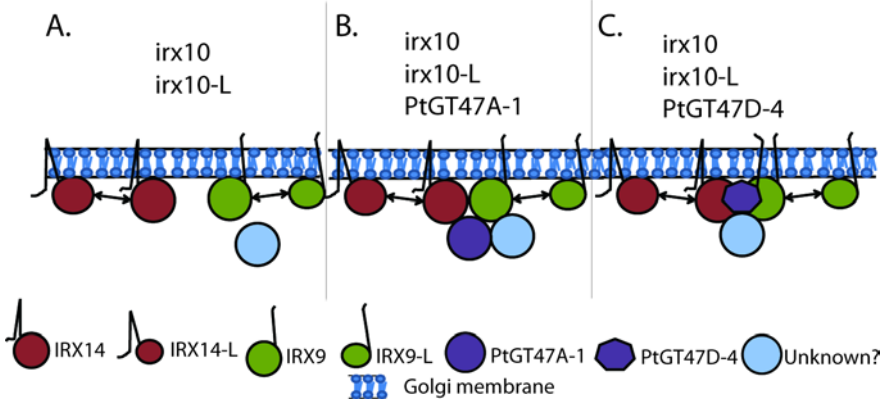

Figure 15. Hypothetical model of the GX biosynthesis complex in the Atirx10irx10-L *double mutant plants (A) and the* Atirx10irx10-L *double mutant plants complemented with the* Populus PtGT47A-1 *(B) and* PtGT47D-4 *(C) genes respectively.*

In Arabidopsis, AtIRX10-L has also been proven less efficient in GX biosynthesis than AtIRX10 (Brown *et al.*, 2009; Wu *et al.*, 2009). Together these results suggest a slightly different function of some of the IRX10 gene family members. Although the high degree of sequence similarity indicates a similar function, the general expression patterns of *PttGT47D1/4* and *AtIRX10-L* differ from the almost exclusive xylem-specific expression of *PttGT47A-1* and *AtIRX10*. This suggests that the IRX10 gene family has gone through a subfunctionalisation event. It is possible that the general expression pattern of *PttGT47-D* and *IRX10-L* reflects involvement in primary cell wall xylan. The

hypothesis that the *IRX10* gene family has gone through a subfunctionalisation event is further supported by data presented in IV.

The lack of phenotype exhibited by the *Populus* RNAi plants was surprising. The two studies published so far (Lee *et al.*, 2009b; Li *et al.*, 2011) have discussed *Populus* proteins putatively involved in the biosynthesis of the xylan reducing end tetrasaccharide. One possibility is that the plant is able to cope with GX that is reduced in length but has an intact reducing end tetrasaccharide, perhaps by producing more polysaccharides, while if the reducing end tetrasaccharide is severely affected, the plant might not be able to compensate for it in the same way. Alternatively, plants with severely affected GX chain length might not be viable and did thus not survive the transformation process. In fact, the low number of lines obtained (36) from six independent transformations clearly suggests that severely affected transformants were not able to go through the normal selection procedure and that the transformation and regeneration process negatively selected for plants with high down regulation. The results obtained in this study for the GT47A and D RNAi lines could thus reflect a difference in importance for the plant cell wall to have a fully elongated backbone compared to an intact reducing end sequence.

Finally, a small but statistically significant reduction in stem height could be seen for all three lines when grown 6 weeks in soil, and a small reduction in stem width could be seen for one of the lines. In the two previously published studies regarding GX biosynthesis in *Populus*, plant materials were more than 5 months old (Lee *et al.*, 2009b; Li *et al.*, 2011). It is possible that the growth effect in our study would have been more pronounced if the plants were grown for longer time.

5.3 Analysis of GX biosynthesis candidates in *Physcomitrella* with focus on the IRX10 gene family member *PpGT47D* (Paper IV)

Not until recently, xylan has been detected in bryophytes (Carafa *et al.*, 2005; Moller *et al.*, 2007; Kulkarni *et al.*, 2012), and very little is known about bryophyte xylan biosynthesis. In paper IV, a search was performed in the genome of the bryophyte *Physcomitrella* for homologs for all *Arabidopsis* proteins known to be involved in GX biosynthesis. Two homologs were found for AtIRX9/IRX9-L, three homologs for AtIRX14/IRX14-L, one homolog for AtIRX10/IRX10-L, and three homologs for AtFRA8/F8H, but no homologs were found for AtIRX8 or AtPARVUS. The highly conserved homolog to the *Arabidopsis* IRX10 and IRX10-L proteins was named PpGT47D. *PpGT47D* was

introduced into the *Arabidopsis irx10irx10-L* double mutant background for complementation studies. The PpGT47D expression pattern in *Physcomitrella* was investigated with the GUS reporter system and the gene was knocked out in *Physcomitrella*.

The complemented *Atirx10irx10-L* plants displayed increased rosette sizes and improved bolting capacity compared to double mutant plants, implying that the function of *Arabidopsis* IRX10 and IRX10-L and *Physcomitrella* PpGT47D partly overlap. In contrast, although a trend towards increased GlcA content could be noted, the overall monosaccharide composition of the cell wall fraction from the stem of complemented *Arabidopsis* plants did not differ in relative amounts from non-complemented plants. This implies that the improvement in phenotype was due to an overall increase in secondary cell wall deposition. It is possible that a small amount of xylan is produced in the complemented plants but that the increase is below the detection limit of the experiment. Xylan binds to lignin and cellulose and is probably important both for holding the hemicellulosic network together and for the impregnation of lignin in the secondary cell wall which results in impermeability of the secondary cell walls. A small amount of produced xylan would then be enough for the plant to improve water conducting properties, but not enough to restore the plant to a wild-type appearance. It is possible that the same type of event as described earlier for the PtGT47D-4 protein (Fig. 15C), in which mislocalisation and/or altered structure of the PpGT47D may combine to produce only a small amount of a functional GX synthesis complex. Alternatively, a complex with low activity is formed in the partially complemented *Arabidopsis* plants. The occurrence of increased secondary cell wall deposition was supported by stem sections stained with safranin, where the *irx10/irx10-L* double mutant plants only showed staining in the cell corners but the complemented plants had staining around the whole cells (Fig. 16).

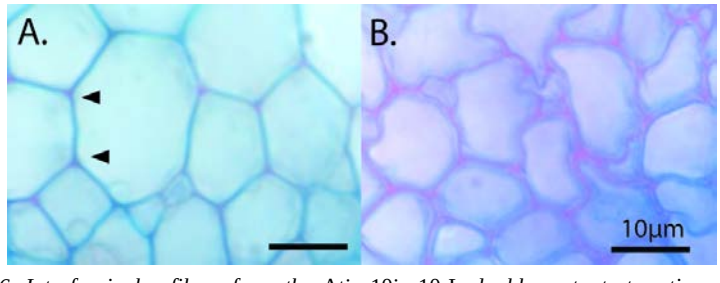

Figure 16. Interfascicular fibers from the Atirx10irx10-L *double mutant stem tissue (A) with lignification only in the cell corners (arrowheads) and the double mutant plants complemented with* PpGT47D *(B), displaying secondary cell wall deposition around the cells.*

Another explanation is that the PpGT47D protein could have an alternative main function than the *Arabidopsis* IRX10 protein. Previous studies (I and III) have indicated that the IRX10 gene family has gone through a subfunctionalisation event, and that some of the proteins (IRX10-L and PtGT47D-1/4) may be involved in primary cell wall biosynthesis. Furthermore, the clustering of PpGT47D with AtIRX10-L and PtGT47D-1/4 indicates that they are closer to the original form of the protein.

Gametophores from the *Physcomitrella* knock-out plants were investigated with monosaccharide analysis, but revealed no difference compared to wild-type in sugar content. From this study it is not possible to assign a definite enzymatic function to the PpGT47D protein. It is interesting to note though, that previous studies have suggested that NpGUT1, another member of the IRX10 family of genes, is involved in RGII biosynthesis (Iwai *et al.*, 2002). Both xylan and RGII are minor constituents of the bryophyte cell wall, and structural alterations could perhaps be under the detection limit in the cell wall of *PpGT47D* knock-outs. Thus, xylan and RGII are perhaps the two best candidate polymers for further studies to elucidate the function of the PpGT47D protein. One hypothesis to be tested is if the original protein could synthesise primary cell wall RGII and/or xylan and that a neo-or sub-functionalisation event during the evolution has allowed some of the members to become more specialized for xylan synthesis in angiosperms.

In conclusion, this study highlights that the GX backbone biosynthesis machinery is partly conserved across embryophytes, but also that there has been specialisation amongst GTs. No enzymatic function could be assigned to the *Physcomitrella* GT47D protein, but information regarding the evolution of the *IRX10* gene family provides a new starting point to push the investigation of xylan related GTs further.

6 Conclusions and Future Perspectives

6.1 Paper I, II and IV

Analysis of *Arabidopsis* mutants affected in xylan synthesis has provided valuable insight into the enzymatic machinery necessary for its biosynthesis. In paper I, the Arabidopsis IRX10 and IRX10-L proteins were proven to be involved in GX biosynthesis. Data presented in paper II further contributed to the identification of the *IRX9-L* and the *IRX14-L Arabidopsis* genes, and confirms their involvement in GX biosynthesis. However, three major questions are still to be answered concerning GX synthesis in *Arabidopsis*;

1. The identification of functional homologs to IRX9, IRX10, IRX14 and FRA8 (I and II) was an important step towards unraveling the GX biosynthesis machinery in *Arabidopsis*, but the reason for the maintenance of the closely related pairs of genes *IRX9/IRX9-L*, *IRX10/IRX10-L*, *IRX14/IRX14-L* and *FRA8/F8H* is currently unclear. Results presented in this thesis (I, III and VI) suggest that the IRX10 gene family has undergone a sub- or neofunctionalisation event. Additional work on the evolution of xylan and the xylan biosynthesis machinery could contribute significantly to answering this question. It is especially interesting that there is only one IRX10 related gene in *Physcomitrella* which indicates that a functional homolog for IRX10 gene family members is not crucial in bryophytes.
2. Data obtained in this thesis (II), as well as the work of other groups (York & O'Neill, 2008; Keppler & Showalter, 2010; Lee *et al.*, 2010; Lee *et al.*, 2012), suggests that the IRX9, IRX14 and perhaps IRX10 proteins form part of a GX backbone synthesis complex. Identification of all protein members of such a complex would represent a significant breakthrough in the field. Strong evidence has been brought forward that IRX9 and IRX14 work cooperatively in GX backbone biosynthesis, but the exact nature of this

interaction is not yet elucidated. A pull-down experiment would be a plausible way to complement today known data regarding the exact composition of the GX backbone synthesising complex.

3. Further, it would be interesting to compare the GX backbone synthesising complex and its evolution with the cellulose rosette complex. Can it be so that the existence of homologs in *Arabidopsis* to the known members of a xylan biosynthesis complex reflects the presence of one primary and one secondary cell wall xylan biosynthesis complex analogous to the primary and secondary cell wall rosette complexes found for cellulose? Can the uneven number of homologs identified in *Physcomitrella* (IV) reflect the lack of a secondary cell wall xylan complex, or perhaps a different form of xylan biosynthesis in bryophytes similar to the cellulose biosynthesis machinery found in some bacteria and algae?
4. Many groups have tried to demonstrate enzymatic activity for any of the proteins involved in GX biosynthesis, but with poor success. So far only indirect evidence in the form of microsome experiments have been successful. The identification of a presumed complex and the identification of all its protein members is most probably a necessary first step in order to design functional enzymatic assays and assign the GTs involved in GX synthesis specific enzymatic functions.

In *Physcomitrella*, it would clearly be interesting to extend the knowledge about xylan and xylan biosynthesis to the diploid sporophyte generation, which from one point of view could be considered more likely to share some qualities with the angiosperm diploid generation.

6.2 Paper III

GX is one of the most abundant polymers in dicot wood, a raw material of major industrial interest. It is therefore very important to start to elucidate how much of the knowledge gained in the herbaceous species *Arabidopsis* can be applied in woody plants. Until now, the molecular machinery synthesising GX in *Populus* has just started to be unraveled. Transcriptomics data, complementation studies and RNAi experiments have provided good indications that the GX enzymatic machinery is highly conserved between *Arabidopsis* and *Populus*. The identification of a GX biosynthesis complex and success to obtain functional enzymatic assays in *Arabidopsis* would clearly open for further testing of this hypothesis. Another important step would be to produce *Populus* knock-outs, or as this is not yet possible, take the advantage of inducible systems. Inducible systems would make it possible to overcome a

presumed regeneration problem during the transformation procedure and obtain knock-downs of *GT47A* and *GT47D* genes where the down-regulation is more than the 75% obtained in this study.

Furthermore, all measurements mentioned above have been done at the DNA or RNA level, but have not addressed the protein directly. For example the RNAi lines were investigated for reduced gene expression based on the mRNA level which is measured using qPCR, without taking into account the possibility that the regulation of cell wall biosynthesis can be at the protein level. Perhaps the remaining 25% of mRNA transcripts in the GT47KD lines are sufficient to maintain normal GX production under the greenhouse conditions used in this study, where for example light conditions (in this experiment up to about 1/10 of outside maximum) might provide bigger restrictions for optimal growth than the access to GX biosynthesising enzymes. Profound transcriptome remodeling has been suggested to be a fundamental difference in growth strategy between the annual plant *Arabidopsis* and the perennial *Populus* plants (Geisler-Lee *et al.*, 2006). It is likely that other types of regulation for example at the protein level might exist between the two species. In order to take the knowledge one step further, it is therefore necessary to also map the expression of the GX biosynthesis proteins with for example western blot experiments to confirm qPCR expression data, especially for RNAi lines.

6.3 Epilogue

In fact, my work has provided valuable information which has led to new hypotheses and models for xylan biosynthesis. It is obvious that more studies are necessary to prove the enzymatic activity of the IRX10 family of proteins, as well as the enzymatic activity of the rest of the GX biosynthesis proteins. Just before the submission of this thesis to print, a paper was published which shows that IRX9 and IRX14 work together in a complex and possibly can work independently of IRX10. This raises new questions about the possible composition of such complex and the specific role of the IRX10 family of proteins. Clearly, much still remains to be unraveled regarding the biosynthetic machinery of GX.

The possibility to tailor wood raw material for specific purposes has long been sought for in order to improve processing and decrease the use of chemicals within the industry. The work presented in this thesis has shown that the possibility to directly apply knowledge gained in *Arabidopsis* cell wall mutants to *Populus* plants is perhaps not as straight forward as previously

thought. It is likely that the methods available today are not yet ultimately developed for cell wall studies in a perennial model organism such as *Populus*, which probably has plasticity and ability to adjust to environmental and physiological circumstances as the most important traits in order to acclimatise to a changing environment. Despite this, in the next few years testing of the hypothetical models presented above is likely to provide methods to manipulate the composition of secondary cell walls also in *Populus*. Hence, specific tailoring of the raw materials required for a wide range of industrially important processes might be reality but discoveries on the way will hopefully also open for new application areas of the cell wall and its components.

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Glucuronoxylan (GX) is the second most abundant polysaccharide in the secondary cell walls of angiosperms. The thesis is based on studies performed in *Physcomitrella patens*, *Arabidopsis thaliana* and *Populus* plants with the aim to increase the understanding of glucuronoxylan synthesis and with main focus on the role of the *IRX10* gene family. Work described in this thesis suggests that the function of the IRX10 family of proteins is partly conserved between *Arabidopsis, Physcomitrella* and *Populus*.

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