Xylan Biosynthesis and Modification

Characterisation of a Glycosyltransferase and a Glycoside Hydrolase in Hybrid Aspen

Christine Ratke

Faculty of Forest Sciences

Department of Forest Genetics and Plant Physiology

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Cover: Stem cross-section of a 2-month-old greenhouse grown hybrid aspen (*Populus tremula* x *tremuloides*) including bark, vascular cambium and xylem. Blue staining indicates GUS expression driven by the *GT43B* promoter (C. Ratke)

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Abstract

Wood is an important renewable material used by humans for a variety of downstream applications. The basic subcellular structure in wood is the cell wall, mainly consisting of the cross-linked polymers cellulose, hemicellulose, and lignin. Xylan is the main hemicellulose found in angiosperm wood, and its biosynthesis and effects on wood properties in hybrid aspen (*Populus tremula* x *tremuloides*) were the object of the present study. We identified a glycoside hydrolase in developing wood, *Ptxt*Xyn10A—a previously suggested xylanase—and found that it rather has xylan transglycosylase activity. This xylan transglycosylase probably rearranges xylan chains during secondary wall deposition. Interestingly, it affected the orientation of cellulose microfibrils, giving new insights into the interaction of cell wall polymers and their control during deposition into the wall. To study genes involved in secondary wall formation and to modify wood properties using transgenic approaches, it is essential to efficiently target transgene expression to the secondary wall forming cells. We isolated and tested a promoter, *pGT43B*, which proved to be very effective in altering gene expression and in generating intended chemical modifications in woody cells. We applied this *GT43B* promoter to modify xylan acetylation and polymerisation in hybrid aspen wood. Furthermore, it enabled the identification of the sucrose transporter SUT3, elucidating how sucrose is transported from ray to developing fiber and vessel cells. Glycosyltransferases (GTs) are enzymes involved in polysaccharide biosynthesis. We characterised the xylan biosynthetic *GT43* gene family in hybrid aspen and found that reduced *GT43* expression levels positively affected overall plant growth, wood mechanical strength, and saccharification efficiency, indicating potential applications for engineering bioenergy feedstocks. We conclude that cell specific modification of xylan properties in hybrid aspen can alter cellulose deposition and result in activation of cambial proliferation which has a positive impact on overall biomass yield and properties for downstream woodutilisation.

Keywords: Wood formation, secondary cell wall, xylan biosynthesis, *Populus*, GT43, Xyn10, xylanase, promoter, genetic engineering

Author's address: Christine Ratke, SLU, Department of Forest Genetics and Plant Physiology, 90183 Umeå, Sweden
E-mail: Christine.Ratke@slu.se

"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."

Albert Einstein, Relativity: The Special and the General Theory

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 Marta Derba-Maceluch, Tatsuya Awano, Junko Takahashi, Jessica Lucenius, **Christine Ratke**, Inkeri Kontro, Ondrej Kosik, Ryo Tanaka, Anders Winzéll, Åsa Kallas, Joanna Leśniewska, Tarek Elhasi, Fredrik Berthold, Peter Immerzeel, Tuula T. Teeri, Ines Ezcurra, Paul Dupree, Ritva Serimaa and Ewa J. Mellerowicz. Suppression of a xylan transglycosylase PtxtXyn10A affects cellulose microfibril angle in secondary wall in aspen wood. *Manuscript.*

II **Christine Ratke**, Prashant Pawar, Vimal Kumar Balasubramanian, Mathilda Lönnäs Duncranz, Ines Ezcurra and Ewa J. Mellerowicz. *Populus* GT43 family gene members show differential expression and include promoters useful for wood modification. *Submitted.*

III Amir Mahboubi, **Christine Ratke**, András Gorzsás, Manoj Kumar, Ewa J. Mellerowicz and Totte Niittylä. (2013) Aspen SUCROSE TRANSPORTER 3 allocates carbon into wood fibres. *Plant Physiology* 163 (4): 1729-1740.

IV **Christine Ratke**, Barbara Terebieniec, Sandra Winestrand, Thomas Grahn, Jennifer C. Mortimer, Bastian Schiffthaler, Thomas Ulvcrona, Paul Dupree, Nathaniel R. Street, Leif Jönsson and Ewa J. Mellerowicz. Altering xylan biosynthetic GT43 glycosyltransferases in hybrid aspen leads to increased growth and improved wood mechanical properties and saccharification. *Manuscript.*

Additional publication by the author:

Patent SE1351248-8: Transgenic trees having reduced xylan content. Inventors: Ewa Mellerowicz, Leif Jönsson, **Christine Ratke**, Sandra Winestrand and Barbara Terebieniec. Filed 2013 by SweTree Technologies AB, Umeå, Sweden

The contribution of Christine Ratke to the papers included in this thesis was as follows:

I Bioinformatic analysis, protein extractions and western blotting, preliminary experiments with Xyn10 expression and purification, participation in discussion of experimental design, data analysis and writing.

II Planning, performance and analysis of results concerning bioinformatics, gene expression, promoter cloning, generation of GUS transgenic lines, histochemical GUS staining, cloning of plant expression vector, cloning of RWA-RNAi lines. Writing the manuscript together with EM.

III Preparation of the GT43B promoter vector, analysis of its expression by GUS and revision of the article.

IV Planning, performance and analysis of most experimental work, including: phylogenetic analysis, RNAi cloning, screening of transgenic lines, greenhouse experiments, phenotyping, wood anatomy and sugar analysis. Xylan chain length analysis together with JM and mechanical testing together with TU. X-ray and RNA sequencing data analysis. Writing the manuscript.

Abbreviations

ACL5	ACAULIS5
AGP	Arabinogalactan protein
bHLH	basic helix-loop-helix
BLH	BEL1-LIKE HOMOEODOMAIN
BR	Brassinosteroid
CAZyme	Carbohydrate-active enzyme
CBM	Carbohydrate-binding module
CE	Carbohydrate esterase
CES	CELLULOSE SYNTHASE
DA	Degree of acetylation
DP	Degree of polymerisation
ERF	Ethylene response factors
F8H	FRA8 homolog
FRA	FRAGILE FIBER
GA	Gibberellin
GalA	Galacturonic acid
GH	Glycoside hydrolases
Glc	Glucose
GlcA	Glucuronic acid
GT	Glycosyltransferase
GUS	β-glucuronidase
GUX	Glucuronic acid substitution of xylan
GX	Glucuronoxylan

GXM	Glucuronoxylan methyltransferase
HD-ZIPIII	HOMEODOMAIN-LEUCINE ZIPPERIII
IRX	IRREGULAR XYLEM
LBD	LATERAL BOUNDARY DOMAIN
LCC	Lignin-carbohydrate complex
MC	METACASPASE
MP	MONOPTEROS
MYB	MYELOBLASTOSIS
NST	NAC SECONDARY WALL THICKENING PROMOTING FACTOR
PL	Polysaccharide lyase
REO	Reducing end oligosaccharide
Rha	Rhamnose
RWA	REDUCED WALL ACETYLATION
SCL	SCARECROW-LIKE
SMRE	Secondary wall MYB-responsive element
SND	SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN
SuSy	SUCROSE SYNTHASE
SUT	SUCROSE TRANSPORTER
TBL	TRICHOME BIREFRINGENCE-LIKE
TE	Tracheary element
UGD	UDP-Glucose dehydrogenase
UXS	UDP-Xylose Synthase
VND	VASCULAR RELATED NAC DOMAIN
WOX	WUSCHEL-related HOMEOBOX
XTH	XYLOGLUCAN ENDO-TRANSGLYCOSYLASE/ HYDROLASE
Xyl	Xylose

1 Introduction

1.1 Why Wood Research?

From the beginning of its life, a plant thrives to grow, driven by its need for sunlight and water. The plant body expands to reach favourable habitats and interact with the environment, since the plant cannot move as animals can. Trees grow tall! For keeping their balance, they need to build up solid supportive structures. Structures, which allow their large photosynthetic tissues to reach the sunlight, structures which enable water transport, and structures which protect against biotic and abiotic stresses: woody stems! The stem of a tree consists of two main parts, the internal wood and the external protective bark (Raven *et al.*, 2005). Accumulation of the woody tissue during secondary growth results in substantial expansion of the stem.

Wood has been used by humans since they understood that they can make use of the materials around them (Youngs, 2009). It is utilised as fuel, as building material for a wide range of products, and used more than any other material for construction. Today, new product demands and technologies result in novel and different forms of wood utilisation. There is a general world trend toward increasing wood use, with a worldwide consumption of 2.3 billion m^3 in year 1961 compared to 3.5 billion m^3 (of which half were used as fuel) in year 2007 (Pepke, 2010). The demands of the growing world population challenge supplies of raw materials and disposition of used products. Fossil fuels are limited, food and energy feedstocks are competing and the environment is accumulating dangerous waste products. Political and societal efforts are necessary to find a sustainable solution for feeding, transportation and keeping up "necessary" infrastructures for humanity. Research in general broadens and deepens our knowledge, which is required to make the right decisions. Wood research aims at drawing a more complete picture of wood

formation, wood properties and wood utilisation. Wood research will guide us how we can make the best use of wood in a sustainable manner.

1.2 Wood Formation

The apical meristems at the shoot and root tip are established during plant embryogenesis and give rise to the primary meristems: protoderm, ground meristem and procambium (Raven *et al.*, 2005). These meristems are precursors of primary epidermal, ground and vascular tissues of the primary plant body. Vascular tissue contains the conducting tissues xylem and phloem. Xylem transports water and minerals from the roots; phloem transports assimilate from the leaves (source tissues) to developing tissues (sink tissues).

Primary growth results from cell divisions in the apical meristems. During secondary growth of woody perennials, cell divisions in lateral meristems give rise to the secondary tissues which are responsible for the great lateral expansion of tree trunk and root (Figure 1) (Larson, 1994). There exist two lateral meristems, the vascular cambium and the cork cambium. Meristematic cells in the vascular cambium produce secondary xylem—the wood—to the inside and phloem to the outside. To compensate for the increase in plant circumference and replace the epidermis, a cork cambium develops in older plants, giving rise to cork as the new protective external tissue.

The vascular cambium contains two forms of meristematic cells, fusiform and ray initials (Larson, 1994). Fusiform initials are vertically oriented and several times longer than wide, they give rise to the axial secondary xylem and phloem cells. Ray initials are isodiametric or slightly horizontally elongated. They give rise to ray parenchyma cells, which extend as vascular rays radially through xylem and phloem. These radial rays are mainly transporting assimilates, nutrients and water between phloem and xylem. When fusiform and ray initials produce xylem and phloem, they divide periclinally, which means that the cell plate formed between the new cells is parallel to the stem surface. Cell proliferation based on periclinal divisions increases the diameter of the stem. To compensate this diameter increment with an equivalent increase of initials in the circumference, the meristem initials also divide anticlinally.

Secondary xylem tissue consists of tracheary elements (TE), which are the water conducting cells, fibers, which give mechanical support, and parenchymatic cells (ray cells and axial parenchyma) (Raven *et al.*, 2005). Tracheary element is the collective name for vessel elements (dominating in most angiosperms) and tracheids (dominating in gymnosperms and some angiosperms). They are supported by secondary walls with perforations and/or

pits to enable water transport. At maturity, tracheary elements undergo programmed cell death and lose their protoplasts, which make them very efficient in conducting water (Bollhöner *et al.*, 2012). Vessel elements have brought water transport to perfection; by losing their end walls and aligning end-to-end to long hollow conduits they further reduced mechanical hindrance. The main cell type (55% of wood volume) in angiosperm secondary xylem is xylary fibers, giving mechanical support to the plant body (Panshin & de Zeeuw, 1980; Esau, 1965).

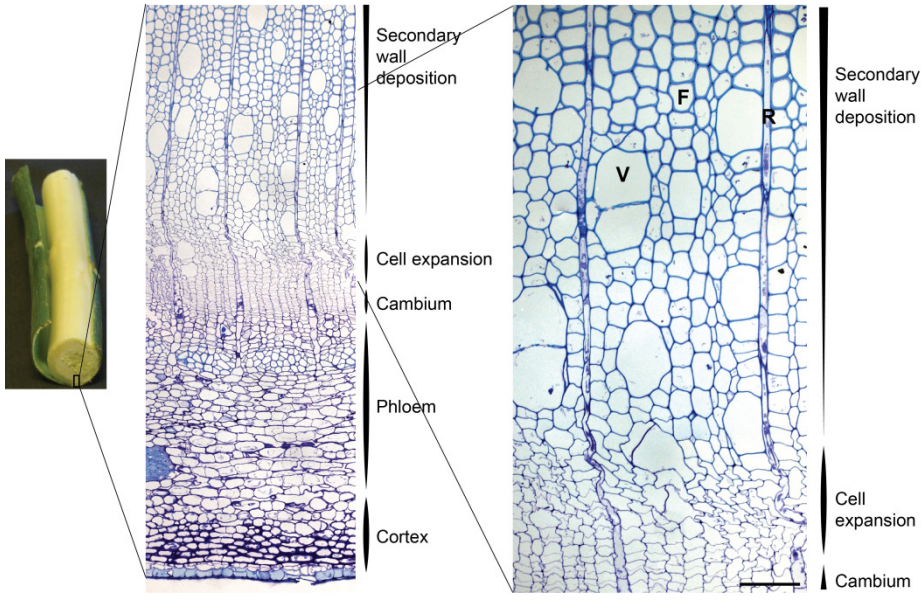

Figure 1. Wood formation. Stem segment of a greenhouse grown hybrid aspen and cross sections containing the vascular cambium and secondary phloem and xylem. Blue colour indicates GUS staining due to activity of GT43 promoter driven GUS. V = Vessel element, F = Fiber, R = Ray, Scale bar 50µm.

Xylary fibers and tracheary elements in the secondary xylem have an evenly deposited, thick secondary wall. In contrast, secondary walls of protoxylem vessels—which are the first-formed vessels of the primary xylem—are deposited in an annular (ring-like) or helical (spiral) pattern. This allows the early primary xylem to be stretched to some extent together with the growing tissue (Raven *et al.*, 2005).

Similar to tracheary elements, fibers undergo programmed cell death at maturity (Courtois-Moreau *et al.*, 2009). A main difference between vessel element cell death and tracheid/fiber cell death is its pace: Vessel elements undergo rapid programmed cell death after a few days, followed by clearing of

the cell contents, which facilitates prompt water transport (Bollhöner *et al.*, 2012). Fibers and tracheids are estimated to live for around one month and their cell-death program begins with the slow disintegration of cellular contents.

1.3 Regulation of Xylem Development

The processes involved in xylem formation—cell division, expansion, secondary wall deposition, lignification and cell death—underlie a strict regulatory network of hormones, miRNAs and transcription factors, integrating the positional information required for xylem differentiation (Figure 2). The balance between cambium maintenance and xylem cell differentiation needs to be tightly regulated to keep tissue homeostasis (Zhang *et al.*, 2014; Hussey *et al.*, 2013; Gorshkova *et al.*, 2012).

Studies using *Arabidopsis* mutants have improved our understanding regarding the hormonal and transcriptional regulatory networks involved in xylem formation. The majority of *Arabidopsis* genes involved in xylem regulation have evolutionary conserved orthologs in woody species like *Populus* (Tuskan *et al.*, 2006). In *Populus,* gene expression analysis along the wood developmental zone confirmed expression of putative genes involved in wood formation (Andersson-Gunneras *et al.*, 2006; Schrader *et al.*, 2004; Hertzberg *et al.*, 2001). Furthermore, several secondary wall biosynthesis regulating factors are functionally conserved between *Arabidopsis* and other angiosperms (Hussey *et al.*, 2013). In the following, mainly the functionally characterised *Arabidopsis* xylem regulatory factors will be presented.

1.3.1 Cambium maintenance

Many plant hormones (auxin, cytokinin, gibberellin, ethylene and jasmonate) stimulate cambial activity (Zhang *et al.*, 2014; Nieminen *et al.*, 2012). Auxin though is the only plant hormone which on its own induces differentiation of tracheary elements in tissue cultures, indicating its key role in xylem differentiation (Fukuda & Komamine, 1980). During vascular development, local auxin maxima define the position of the procambial tissue. This auxin accumulation is generated through a positive feedback loop including *MONOPTEROS (MP)* and the auxin efflux carrier *PIN-FORMED 1* (*PIN1*) (Ohashi-Ito & Fukuda, 2010). MP also directly activates basic helix-loop-helix (bHLH) transcription factors responsible for procambial initiation (Schlereth *et al.*, 2010) and promotes xylem mother cell proliferation by activating transcription factors of the class III homeodomain-leucine zipper (HD-ZIPIII) family (Carlsbecker *et al.*, 2010; Donner *et al.*, 2009; Scarpella *et al.*, 2006).

Another cambial stimulator is the homeobox transcription factor *WUSCHEL-related HOMEOBOX 4 (WOX4)*, which seems to act downstream of auxin signalling (Suer *et al.*, 2011; Ji *et al.*, 2010).

Additional phytohormones often act synergistically together with auxin, which complicates the breakdown of individual regulatory roles. Gibberellins (GAs), for example, stimulate together with auxin cambial cell division, fiber elongation and general xylogenesis (Mauriat & Moritz, 2009; Israelsson *et al.*, 2003; Eriksson *et al.*, 2000). Downstream GA signalling is regulated by the two antagonists DELLA—a master growth repressor—and SCARECROW-LIKE 3 (SCL3), a positive regulator of GA responses (Zhang *et al.*, 2011; Sun & Gubler, 2004).

Brassinosteroids (BR) are another group of phytohormones which are involved in early cambial specification but also in xylem differentiation (Hussey *et al.*, 2013). BRs are suggested to affect vascular differentiation via activation of HD-ZIP III transcription factors (Jung & Park, 2007; Ohashi-Ito *et al.*, 2002) and were shown to affect primary wall cellulose synthase (CesA) expression (Xie *et al.*, 2011).

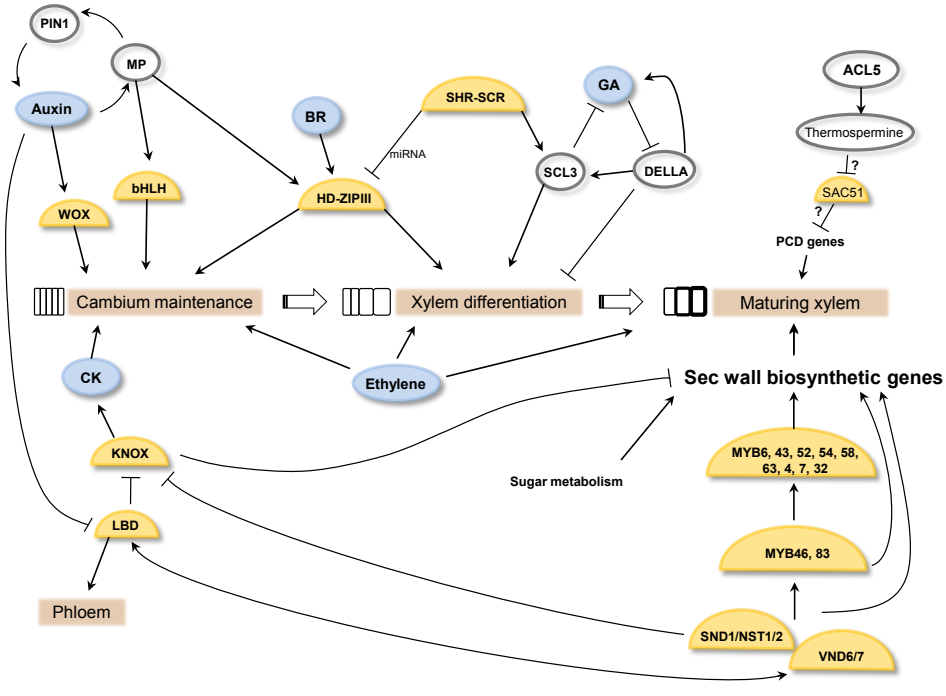

Figure 2. Hormonal and transcriptional control of xylem formation. Blue circles contain plant hormones and yellow half-circles represent transcription factors.

The plant hormone ethylene stimulates cambial cell division (Love *et al.*, 2009) but also seems to play a role in xylogenesis, as it is essential for *in vitro* TE differentiation (Pesquet & Tuominen, 2011). Ethylene further promotes the formation of reaction wood, such as tension wood in *Populus* (Love *et al.*, 2009).

Several KNOX transcription factors promote meristem proliferation and inhibit xylem differentiation (Zhang *et al.*, 2014; Hussey *et al.*, 2013). In *Populus*, the KNOX transcription factors *ARK1 and 2 (ATKNAT1, ATSTM, ATBP)* activate cambial proliferation—possibly via activation of cytokinin—and repress secondary wall deposition (Gorshkova *et al.*, 2012). KNAT7, another KNOX gene, directly represses secondary wall biosynthetic genes and is itself suppressed by SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 1 (SND1) (Hussey *et al.*, 2013). *KNOX* gene expression is furthermore negatively controlled by LATERAL BOUNDARY DOMAIN 1 (LBD1) transcription factors and by BEL1-LIKE HOMOEODOMAIN proteins (BLH4/SAW1/2) (Gorshkova *et al.*, 2012; Yordanov *et al.*, 2010; Kumar *et al.*, 2007).

1.3.2 Control of xylem maturation

When xylem cells have reached their final size they develop secondary cell walls composed of mainly cellulose, hemicellulose and lignin. The secondary wall biosynthetic machinery is activated by an evolutionary conserved multileveled transcriptional network which contains several regulatory circuits between diverse transcription factor families (Zhang *et al.*, 2014; Hussey *et al.*, 2013). Master switches of xylem differentiation are the NAC-domain transcription factors VASCULAR RELATED NAC DOMAIN (VND) 6 and 7 in xylem vessels, and SND1 and NAC SECONDARY WALL THICKENING PROMOTING FACTOR (NST) 1 in fibers. In general, master regulators of secondary wall formation are characterised by their sufficiency of inducing ectopic secondary wall deposition (Mitsuda *et al.*, 2007). NAC-domain master transcription factors invoke cell wall biosynthesis partly through direct activation of biosynthetic genes but also via induction of the down-stream machinery of transcription factors. Direct targets of NAC transcription factors are the redundant master regulators MYELOBLASTOSIS (MYB) 46 and MYB83, with their poplar orthologs PtrMYB2, 3, 20 and 21 (McCarthy *et al.*, 2010; Zhong *et al.*, 2010; Zhong *et al.*, 2007). MYB46/83 again induce activation of the last group of downstream transcription factors, including mainly MYBs but also the KNOX transcription factor KNAT7, as well as of cell wall biosynthetic genes directly (Hussey *et al.*, 2013). Furthermore, the master transcription factors VND6/VND7 regulate themselves in a positive

feedback loop through the ASL/LBD family proteins ASL19/ASL20 (Hussey *et al.*, 2013).

Target gene expression is finally being modulated by binding of the transcription factor to *cis*-regulatory elements in the promoters. Some of the general binding sites of MYB and NAC transcription factors have been described (Hussey *et al.*, 2013). MYB transcription factors for example have been shown to bind AC-rich promoter elements of secondary cell wall biosynthetic genes, also called secondary wall MYB-responsive elements (SMRE) (Zhong *et al.*, 2013; Zhao & Dixon, 2011; Winzell *et al.*, 2010).

After xylem cells have formed their secondary walls, they undergo the final stage of xylem development: programmed cell death. The degradation of cell components and tonoplast rupture finally leaves only the secondary cell wall skeleton (Bollhöner *et al.*, 2012). Several proteases have been shown to be involved in efficient clearing of cell contents during programmed cell death: XYLEM CYSTEINE PEPTIDASE (XCP) 1, XCP2 and METACASPASE 9 (AtMC9) (Bollhöner *et al.*, 2013).

1.4 The Plant Cell Wall and its Components

1.4.1 Primary cell wall

While a plant cell is growing and expanding, the plastic primary wall is deposited beyond the plasma membrane (Albersheim *et al.*, 2011; Fry, 2011). Membrane located protein complexes synthesise cellulose microfibrils, the cell wall "skeleton". The cellulose microfibrils are embedded in the matrix components: the non-cellulosic polysaccharides, including pectins and hemicelluloses (Table 1) (Mellerowicz & Gorshkova, 2012). Primary walls can later become impregnated with lignin, a phenolic polymer, and/or cutin, a polyester. More than 70% of primary wall fresh weight is water, hydrating the cell wall polysaccharides and enabling enzymatic activities (Albersheim *et al.*, 2011). Furthermore, the primary wall contains proteins—structural or enzymatically active—which vary highly in their abundance.

There are major differences between dicotyledon, monocotyledon and gymnosperm hemicellulose composition. Dicots contain mainly xyloglucan in their primary, and glucuronoxylan (GX) in their secondary wall; whereas the monocotyledon grasses include mainly glucuronoarabinoxylan in their primary as well as secondary wall (Scheller & Ulvskov, 2010). The main hemicellulose in gymnosperm primary walls is xyloglucan; their secondary wall contains mainly galactoglucomannan and some glucuronoarabinoxylan.

Table 1. Chemical composition of primary wall (before lignification) and secondary wall layers (S-layers) of dicotyledon fibers in % dry weight (Mellerowicz & Gorshkova, 2012).

Polymer	Primary wall	Secondary wall
Cellulose	30-40	50
Pectin	~40	Minor
Lignin	-	10-20
Protein	1-10	Minor
Hemicelluloses:		
Xyloglucan	20	Minor
Xylan	5	20-30
(Gluco)mannan	3	5

1.4.2 The secondary cell wall

In addition to the primary wall, some plant cells deposit a thick secondary cell wall when they have reached their final size (Mellerowicz & Gorshkova, 2012). The rigid secondary wall gives mechanical support to the individual cell and accordingly to the whole tissue. As wood is largely composed of xylary fibers with very thick secondary walls, those walls are thus the basis of the huge applicability of wood. In contrast to the primary wall, the secondary wall hardly contains any pectin and xyloglucan, instead it includes 20-30% xylan (Table 1) and more cellulose (Mellerowicz & Gorshkova, 2012; Fry, 2011; Scheller & Ulvskov, 2010). Secondary walls are impregnated with the polyphenolic lignin, which increases mechanical strength and impermeability. Lignin accounts for 10-20% of secondary walls in *Populus* wood (Mellerowicz & Gorshkova, 2012).

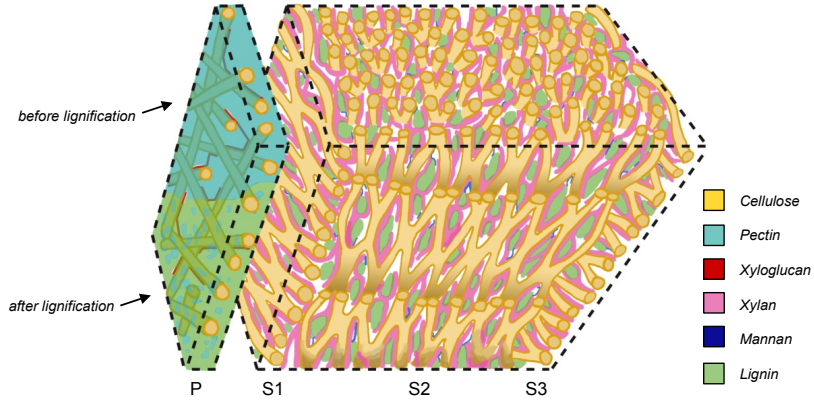

Figure 3. The cell wall and its polysaccharide network. Illustrated are primary wall (P) and the secondary wall layers S1, S2 and S3 (Mellerowicz & Gorshkova, 2012).

The secondary cell wall is much thicker than the primary wall and consists of several layers: the S1, S2 and S3 layers. Cellulose microfibrils have different orientations in the individual wall layers (Mellerowicz *et al.*, 2001). Primary wall cellulose microfibrils are randomly or longitudinally arranged, enabling cell expansion. In contrast, the first secondary wall layer deposited, the S1 layer, has dense and almost horizontally aligned microfibrils—limiting the further radial expansion of the growing xylem cell (Mellerowicz *et al.*, 2001). Following the relatively thin S1 layer, a thick S2 layer is formed, which is characterised by more longitudinally arranged cellulose microfibrils (low microfibril angle). The S2 layer of the secondary cell wall is the biggest fraction in xylary fiber walls and accordingly accounts for the bulk woody biomass. At last, the S3 layer with once again more horizontally arranged microfibrils is deposited. The control of cellulose microfibril deposition seems to involve cytoskeletal microtubules (Paredez *et al.*, 2006). Microtubules are oriented parallel to microfibrils and their disruption disturbs microfibril biosynthesis and cell wall patterning.

1.4.3 Cellulose

Cellulose is a relatively simple linear polymer composed of β-(1→4)-linked glucose subunits. Cellulose can be chemically purified as it is left in the insoluble wall material after matrix polysaccharide extraction with sodium hydroxide (Fry, 2011). The individual cellulose chains have a strong tendency to associate via hydrogen bonding of their hydroxyl groups (Pu *et al.*, 2008). This bonding of individual glucan chains results in aggregation into a highly ordered, crystalline structure: the cellulose microfibril (Pu 2007). The crystalline microfibril structure is regularly interrupted by regions with a lower order of aggregation, which are called amorphous regions. Amorphous microfibril regions are easily accessible to hydrolytic enzymes, whereas crystallinity is thought to have a negative effect on enzymatic hydrolysis (Hu *et al.*, 2012). Generally, cellulose microfibril orientation and organisation strongly affect cell shape, growth and mechanical cell wall properties (Albersheim *et al.*, 2011).

Cellulose is synthesized by plasma membrane embedded enzymes and deposited directly into the cell wall. In contrast, hemicelluloses and pectins are assembled by membrane-associated enzymes in the Golgi apparatus (Johansen *et al.*, 2006). Cellulose chains have a degree of polymerisation (DP) of more than 300 sugar residues—up to several thousand—whereas hemicellulose chains are shorter with a chain length of 50 – 300 subunits (Pu *et al.*, 2008; Klemm *et al.*, 2005). The individual cellulose chains of a single microfibril need to be initiated, elongated and aggregated into the microfibril structure

(Albersheim *et al.*, 2011). Cellulose synthases (CesAs) are arranged in the so called “rosette” cellulose synthase complex, composed of six hexagonally arranged particles. Each rosette complex synthesizes one elementary about 3nm thick microfibril, generally consisting of 20-30 glucan chains (Somerville, 2006; Saxena & Brown, 2005). The rosette complex is translocated in the plane of the plasma membrane driven by cellulose polymerisation and crystallisation, which becomes visible as a straight trajectory when following fluorescently labelled CesAs. The direction of those cellulose biosynthetic trajectories coincides with the orientation of cortical microtubules (Paredez *et al.*, 2006). Distinct sets of CesA proteins seem to be responsible for cellulose deposition in primary walls (CesA1, -3 and -6) and secondary walls (CesA4, -7 and -8), which has been suggested based on genetic and biochemical studies in *Arabidopsis* and expression profiling in, amongst others, developing *Populus* xylem (Kumar *et al.*, 2009).

1.4.4 Pectin

Pectins are complex, branched polysaccharides with a high galacturonic acid (GalA) content, abundant in the primary cell wall (Fry, 2011; Mohnen, 2008). There are four types of pectic polysaccharides, homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. Typical for pectins is their extractability with chelating agents such as EDTA.

1.4.5 Hemicelluloses

The hemicelluloses xyloglucan, xylan and mannan, are non-cellulosic wall polysaccharides which hydrogen-bind to cellulose (Fry, 2011). In this way they are connecting adjacent cellulose microfibrils and are therefore also called ‘crosslinking glycans’. Hemicelluloses are more complex than cellulose in the way that they are branched and that they can contain different side groups and modifications (Pu *et al.*, 2008).

Hemicelluloses can be extracted with chaotropic agents such as sodium hydroxide (Fry, 2011). Chaotropic agents in general destabilize and solubilize non-covalent bonds in macromolecules, for instance hydrogen bonding and attractive forces (van der Waals’ interaction) between hydrophobic regions. The high pH ionizes the hemicellulose hydroxyl groups, they become negatively charged and repel each other. Moreover, all ester-bonded acetyl groups are stripped off.

1.4.6 Xylan

Polysaccharides are generally described based on the sugars building up their backbone; xylan for instance entitles a backbone consisting of xylose subunits.

Further side chains and modifications are added to the name, acetylglucuronoxylan for example defines a xylan backbone containing glucuronic acid (GlcA) as well as acetyl side groups.

Glucuronoxylan—the major hemicellulose in the dicotyledon secondary wall—consists of a β-(1→4)-linked xylosyl residue backbone (Figure 4). On the C-2 of on average every eighth xylose residue a glucuronic acid group attaches (Ebringerová, 2005). Glucuronic acid side groups are frequently methylated with a ratio of GlcA to 4-O-MeGlcA about 1:2 in *Arabidopsis* xylan (Peña *et al.*, 2007). 70% of the backbone xylose residues are O-acetylated at C-2 and/or C-3. Glucuronoxylans contain a specific oligosaccharide at their reducing end consisting of β-Xyl-(1→3)-α-Rha-(1→2)-α-GalA-(1→4)-Xyl (Peña *et al.*, 2007). This sequence is evolutionarily conserved in woody and herbaceous dicot plants and has been suggested to be either a primer or a terminator of xylan biosynthesis (York & O'Neill, 2008).

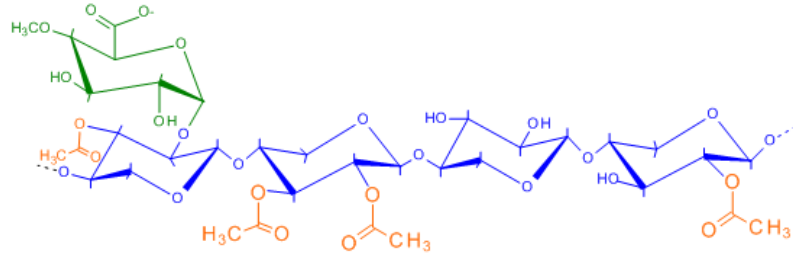

Figure 4. Glucuronoxylan structure. Colour coding of the sugars and decorating groups: xylose backbone = blue, glucuronic acid side group with methyl group (CH_3) = green, O-acetyl group = orange. Image kindly provided by Prashant Pawar.

1.4.7 Lignin

Lignin is the second most abundant organic compound on earth (Raven *et al.*, 2005). In contrast to cellulose and hemicellulose, it is usually not a linear polymer composed of sugar subunits, but an amorphous, three-dimensional phenolic polymer. Lignin is made from phenylpropane monolignols (the cinnamyl alcohol monomers *p*-coumaryl, coniferyl, and sinapyl), which are synthesized in the cytoplasm from phenylalanine. The monolignols are secreted followed by polymerisation in the apoplast (Albersheim *et al.*, 2011).

Lignification of the cell wall starts while the cell is alive and continues after cell death (Pesquet *et al.*, 2013). Lignin replaces the aqueous phase of the cell wall matrix with a hydrophobic polyphenolic polymer. This adds compressive strength and stiffness to the cell wall and is therefore believed to have played a major role in the evolution of terrestrial plants (Raven *et al.*, 2005). Unlignified

walls have relatively high tensile strength, but lignification increases their ability to withstand compressive forces. This allowed land plants to grow taller and support large photosynthetic tissues. Furthermore, strengthening of the cell walls facilitated water transport, as strong water conducting cells are able to mechanically withstand the tension generated by the transpiration stream. Lignification also waterproofs the cell walls which further facilitates water transport.

Lignin strongly hinders accessibility of polysaccharides to hydrolytic enzymes and its reduction is therefore a main target trait to improve in plant feedstocks for biofuel production (Yang *et al.*, 2013). Unfortunately, major lignin reductions are detrimental for overall plant growth (Voelker *et al.*, 2010; Franke *et al.*, 2002).

1.5 Hemicellulose Biosynthesis

1.5.1 Sugar precursors

Sugar precursors for cell wall polymer biosynthesis originate ultimately from photosynthetic carbon fixation. In the photosynthetic source tissues sugars are converted into sucrose—a glucose-fructose disaccharide—for transport to sink tissues via the phloem. In the photosynthetic source tissue, sucrose is loaded into the phloem either via the apoplasm using membrane embedded transporters—which is the case for *Arabidopsis*—or symplasmically through plasmodesmata, as seems to be the case in *Populus* (Braun, 2012; Davidson et al., 2011; Fu et al., 2011). After long-distance transport in the phloem, sucrose is unloaded into the rays, which are responsible for sucrose storage as well as radial transport to the developing xylem cells (Langenfeld-Heyser, 1987). In *Populus*, phloem sucrose unloading into ray cells is believed to occur passively through plasmodesmata, in analogy to phloem loading (Sauter & Kloth, 1986). In contrast, the following sucrose transport from ray cells into growing xylem cells does not appear to be symplasmic, as *Populus* lacks plasmodesmatal connections between rays and developing vessels and fibers (Sokołowska & Zagórska-Marek, 2012). Thus, to enter the developing xylem cell, sucrose needs to pass both ray and xylem cell plasma membranes, which requires membrane embedded sucrose transporters (SUT).

In the developing wood cells, sucrose is converted to sugar nucleotides which are the activated sugar donors for glycan synthesis (Bar-Peled & O'Neill, 2011; Seifert, 2004). Many different nucleotide sugar donors are required for biosynthesis of the diverse cell wall polysaccharides. The majority of nucleotide sugars are sequentially converted from sucrose via UDP-glucose (UDP-Glc) by nucleotide sugar interconverting enzymes (Figure 5). Most

interconverting enzymes are soluble cytosolic proteins; but some enzymes are Golgi membrane bound with the catalytic domain facing the Golgi lumen. Examples for cytosolic enzymes are sucrose synthase (SuSy), which generates UDP-Glc from sucrose (Bar-Peled & O'Neill, 2011), and UDP-glucose dehydrogenase (UGD) which converts UDP-Glc into UDP-glucuronic acid (UDP-GlcA) (Seifert, 2004).

Interestingly, there are two ways that UDP-xylose (UDP-Xyl) can be synthesised from UDP-GlcA by UDP-xylose synthase (UXS): either via a cytosolic or via a Golgi-membrane-bound isoform (Harper & Bar-Peled, 2002; Kobayashi *et al.*, 2002). It is unclear why different enzyme isoforms are synthesising the same nucleotide sugars in separate compartments, but one potential explanation could be a control mechanism at the metabolite level (Bar-Peled & O'Neill, 2011). An example off such a metabolite control mechanism is that UDP-Xyl has been shown to inhibit UXS activity.

Nuleotide sugar transporters are membrane spanning proteins that translocate the cytoplasmically synthesized nucleotide sugars into the Golgi where they are used as substrates during polysaccharide biosynthesis (Albersheim *et al.*, 2011).

During cell wall polysaccharide biosynthesis, glycosyltransferases highly depend on those nucleotide sugars as substrates. Hence, the control of nucleotide interconverting enzymes has been suggested to be one mechanism to control polymer biosynthesis (Seifert, 2004).

1.5.2 Glycosyltransferases

Hemicelluloses are assembled by Golgi-membrane-bound glycosyltransferases (Figure 5). This is distinct to cellulose biosynthesis, where plasma membrane-embedded glycosyltransferases directly synthesize the glucan chains into the cell wall (Johansen *et al.*, 2006). After hemicellulose biosynthesis in the Golgi lumen, the polysaccharides are transported in secretory vesicles to the plasma membrane and integrated into the apoplastic cell wall network.

Glycosyltransferases are membrane-associated enzymes that catalyse the formation of a specific glycosidic bond between the sugar from a nucleotide sugar donor and an acceptor glycan chain. Some glycosyltransferases are type II integral membrane proteins, which are characterised by a catalytic C-terminal domain extending in the Golgi lumen, a flexible stem region and a single transmembrane domain (Oikawa *et al.*, 2013). Glycosyltransferases belong to the ‘Carbohydrate-Active enZyme’ (CAZymes), which have been classified into families based on amino acid sequence similarities (www.cazy.org; (Lombard *et al.*, 2014). CAZymes are modular proteins and group into the following CAZyme families: glycoside hydrolases (GH),

glycosyltransferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and carbohydrate-binding modules (CBM). Plant genomes contain far more CAZymes than mammalian genomes, thereby displaying the diversity of enzymatic activities necessary to produce and modify the plethora of complex plant carbohydrates. Gene duplications during angiosperm evolution resulted in about 1600 CAZy genes in *Populus trichocarpa*, compared to 1000 in *Arabidopsis* (Geisler-Lee *et al.*, 2006). Enzyme activities of glycosyltransferases involve either a retaining or an inverting mechanism depending on the configurations of substrate and reaction product (Sinnott, 1990). A retaining mechanism means that the configuration of the anomeric carbon in the nucleotide sugar donor is retained in the product. Inverting reactions in contrast, are characterised by the inversion of the anomeric carbon configuration from α to β or vice versa.

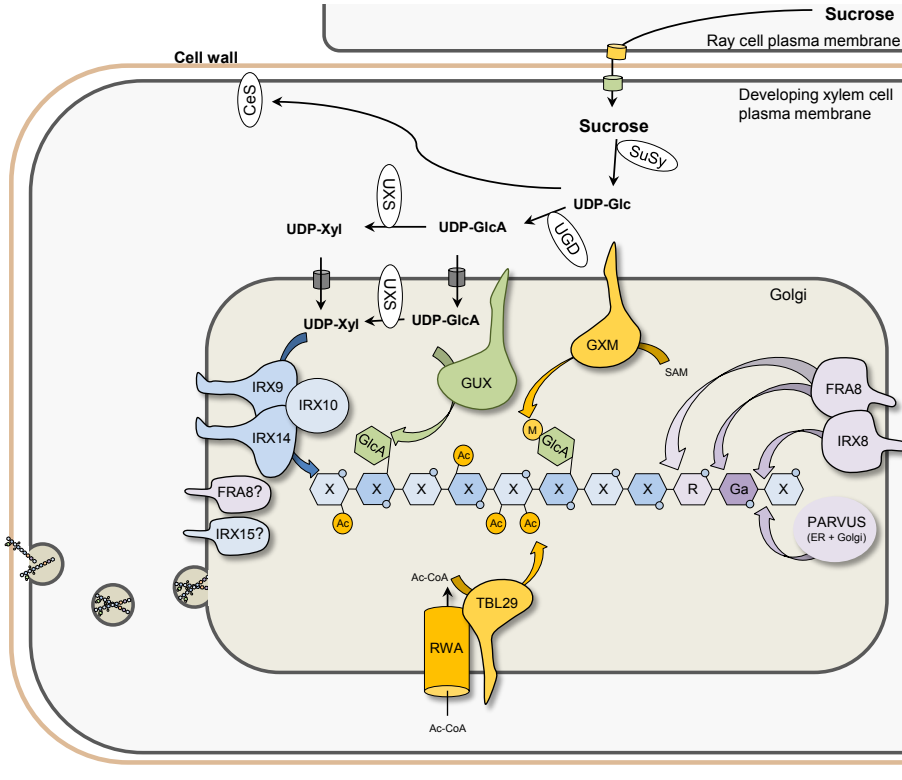

Figure 5. Schematic model of xylan biosynthesis, including sugar precursor allocation, glucuronoxylan biosynthesis in the Golgi lumen and secretion to the apoplasm. Arrows indicate putative catalytic activities.

Polysaccharide backbone biosynthesis generally involves three steps: chain initiation, elongation and termination. *In vitro* studies suggest polysaccharide elongation by tailward growth, which involves the addition of nucleotide sugar donors on the nonreducing polymer end (Albersheim *et al.*, 2011). In enzyme-guided polymerisation systems (such as glycosyltransferase-assisted polysaccharide biosynthesis) the termination appears to be rather uncoordinated (Albersheim *et al.*, 2011). This means that the enzyme-polymer complex simply disintegrates after some time or when the polymer length reduces the complex stability, resulting in the typical variation of product lengths.

The identification and biochemical proving of cell wall biosynthetic glycosyltransferase activities has been challenging (Oikawa *et al.*, 2013). This likely has several reasons: First of all, most glycosyltransferases are membrane bound, which complicates extraction of active enzymes from plant tissues. The second reason is the low abundance of a specific glycosyltransferase in a cell/compartment. Third, glycosyltransferases probably form complexes with other enzymes and often have only limited, if any, activity of their own when expressed in heterologous hosts. Such protein-protein interactions are being suspected between several cell wall biosynthetic enzymes, but have only recently been proven experimentally for a few of them.

1.6 Xylan Biosynthesis

1.6.1 Xylan backbone elongation

Xylan biosynthesis is complex and we are only beginning to understand it (Rennie & Scheller, 2014). One major challenge in the elucidation of xylan biosynthesis has been the severe developmental retardation in xylan mutants (Peña *et al.*, 2007; Brown *et al.*, 2005; Zhong *et al.*, 2005). Another challenge is the unsuccessful attempt of heterologous expression to prove biochemical activity of many candidate genes. Molecular and genetic analysis anyhow demonstrated that the enzymes involved in xylan biosynthesis are xylosyltransferases, glucuronyltransferases, methyltransferases and acetyltransferases.

First candidate genes for secondary cell wall biosynthesis were identified in *Arabidopsis* xylem based on gene expression profiling (Persson *et al.* 2005) and in *Arabidopsis* mutants with collapsed xylem vessels (Brown *et al.* 2005). This xylem phenotype was due to thin secondary cell walls, not able to withstand the negative pressure of the transpiration stream and was called irregular xylem phenotype (irx) (Figure 6).

Xylan biosynthetic candidate genes: IRX9, IRX10 and IRX14

A number of non-redundant proteins required for normal xylan backbone elongation have been identified: IRX9 and IRX14, belonging to glycosyltransferase family 43 (GT43), and IRX10, a GT47 family protein (Table 2) (Brown *et al.*, 2009; Wu *et al.*, 2009; Brown *et al.*, 2007; Lee *et al.*, 2007a; Peña *et al.*, 2007; Brown *et al.*, 2005). Mutations in *IRX9*, *IRX10* or *IRX14* genes in *Arabidopsis* lead to a reduction in cell wall xylan content and xylosyltransferase activity in microsomal fractions. Those putative xylosyltransferases are targeted to the Golgi, the place of hemicellulose biosynthesis. Plants mutated in *IRX9* are severely dwarfed.

The *irx9*, *irx10* and *irx14* phenotypes can be rescued by overexpressing the paralogous genes *IRX9-Like* (*IRX9-L*), *IRX10-L* and *IRX14-L*, respectively (Keppler & Showalter, 2010; Wu *et al.*, 2010; Brown *et al.*, 2009; Wu *et al.*, 2009). The double mutants of each of these functional redundant pairs are more affected than the single mutants and have shorter xylan chains, supporting an essential role of these proteins in xylan backbone elongation. Though IRX9-L, IRX10-L and IRX14-L seem to perform a minor function in xylan biosynthesis as their mutants do not show xylan phenotypes alone and the proteins only partially complement the mutant phenotype of the major xylan biosynthetic genes *IRX9*, *IRX10* and *IRX14*. An interesting finding was that *irx9*, *irx10* and *irx14* mutant phenotypes cannot be rescued by overexpressing xylan biosynthetic genes other than the direct homologs (*IRX9-L*, *IRX10-L* and *IRX14-L*), despite their common involvement in xylan backbone elongation (Wu *et al.*, 2010). Thus, at least one member of the functionally non-redundant gene pairs *IRX9/IRX9-L*, *IRX10/IRX10-L* and *IRX14/IRX14-L* seems to be required for xylan biosynthesis.

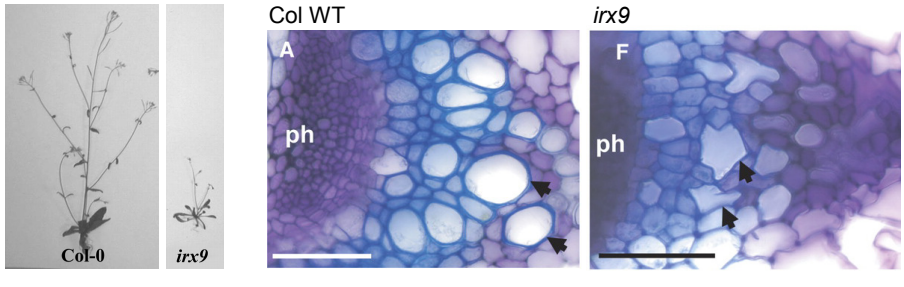

Figure 6. Plant growth and irregular xylem phenotype of a xylan mutant plant (*irx9*) compared to *Arabidopsis thaliana* Columbia wild type (Col). Arrows indicate xylem vessels (Brown *et al.*, 2005).

Interestingly, the *irx14 irx14L* double mutant has the most severe phenotype—with a complete loss of detectable xylan—compared to *irx9 irx9L* and *irx10 irx10L* double mutants which have reduced but detectable xylan contents (Wu *et al.*, 2010; Brown *et al.*, 2009; Wu *et al.*, 2009). The severe phenotypes of *irx14 irx14L* double mutant and *irx9* single mutant suggest those proteins are the essential and rate-limiting components, respectively, in xylan backbone elongation.

GT43 biochemical mechanism

GT43s are inverting glycosyltransferases which can catalyse β-glycosidic linkages using α donor sugars (Anders & Dupree, 2011). During xylan biosynthesis, the configuration of the anomeric carbon in the sugar donor UDP-xylose is inverted from α to β configuration in the xylan polysaccharide. GT43 members could consequently be the enzymes active as β-1,4-xylosyltransferases—which is supported by the mutant xylan phenotypes as well as the co-expression experiments demonstrating xylosyltransferase activity—but the biochemical activity of isolated plant GT43 proteins still remains to be demonstrated. In animals, GT43s have been structurally and biochemically characterised and found to be β-1,3-glucuronyltransferases (CAZy DB, www.cazy.org; (Lombard *et al.*, 2014; Anders & Dupree, 2011). They are involved in glycosylation of proteoglycans, glycoproteins and glycolipids. The enzyme residues important for donor and acceptor recognition in animal GT43s are not conserved in plants, suggesting a divergent biochemical function of plant and animal GT43s.

FRA8

Questions were raised regarding the role of FRAGILE FIBER8 (FRA8) and its homolog F8H. The *fra8* single mutant had longer but fewer xylan chains and therefore was not believed to be involved in backbone elongation (Lee *et al.*, 2009). However, the *fra8* mutant had remaining F8H activity and the *fra8/f8h* double mutant forms only very short xylan chains, which suggests a role in backbone elongation (Wu *et al.*, 2010).

Expression patterns

Expression patterns of the major xylan biosynthetic genes IRX9, IRX10, IRX14 and FRA8 as well as the minor gene IRX14-L in *Arabidopsis* are very similar, with a strong expression in the secondary cell wall forming vasculature (Wu *et al.*, 2010). The minor genes IRX9-L, IRX10-L and F8H exhibit more widespread expression profiles. This supports a common and essential role of IRX9, IRX10, IRX14, IRX14-L and FRA8 in secondary wall deposition.

Table 2. *Arabidopsis* genes involved in xylan biosynthesis

Name	ID	Putative process	Evidence / Mutant phenotype	Reference
IRX9	AT2G37090	Xylan backbone elongation	Reduced XylT activity in microsomes, decrease in chain length and GX content, more REO (more chains), irx phenotype (severe), dwarf, xylose reduction, cooperative XylT activity with IRX14 in tobacco microsomes	Lee et al., 2012b; Wu et al., 2010; Brown et al., 2007; Lee et al., 2007a; Peña et al., 2007; Brown et al., 2005
IRX9L	At1g27600	Xylan backbone elongation	Functional redundant IRX9 paralog	Lee et al., 2010; Wu et al., 2010
IRX14	At4g36890	Xylan backbone elongation	Reduced XylT activity in microsomes, GX content reduced, irx; cooperative XylT activity with IRX9 in tobacco microsomes	Lee et al., 2012b; Keppler & Showalter, 2010; Wu et al., 2010; Brown et al., 2007
IRX14-L	At5g67230	Xylan backbone	Functional redundant IRX14 paralog; severe double mutant lacks xylan!	Keppler & Showalter, 2010; Lee et al., 2010; Wu et al., 2010
IRX10	At1g27440	Xylan backbone elongation	irx phenotype (mild), xylose reduction	Brown et al., 2009; Wu et al., 2009; Brown et al., 2005
IRX10-L	At5g61840	Xylan backbone elongation	Functional redundant IRX10 paralog *(irx10/irx10L* double mutant reduced XylT activity in microsomes)	Brown et al., 2009; Wu et al., 2009
IRX15	At3g50220	Xylan backbone elongation?/ Endomembrane trafficking?	Double mutant reduced GX content, weak irx, shorter chains (SEC), disorganised cell walls	Brown et al., 2011; Jensen et al., 2011
IRX15L	At5g67210			
FRA8/ IRX7	At2g28110	reducing end oligo/backbone?	GX and REO deficient, longer xylan chains, irx phenotype (severe), dwarf	Wu et al., 2010; Brown et al., 2007; Peña et al., 2007; Brown et al., 2005; Zhong et al., 2005
F8H/ IRX7L		reducing end oligo/backbone?	functional redundant FRA8 paralog	Wu et al., 2010; Lee et al., 2009
Parvus/ GATL1	At1g19300	reducing end oligo, pectin?	GX and REO deficient, irx, dwarf	Brown et al., 2007; Lee et al., 2007b; Lao et al., 2003
IRX8/ GAUT12	At5g54690	reducing end oligo + pectin?	GX, REO and HG deficient; irx phenotype (severe), dwarf	Brown et al., 2007; Peña et al., 2007; Persson et al., 2007; Brown et al., 2005
GUX1	At3g18660	glucuronic A side chain	GlcAT activity after purification of transient overexpressed protein in *N. benthamiana*	Rennie et al., 2012; Mortimer et al., 2010
GUX2	AT4G33330	glucuronic A side chain?	Gux1/2 double mutant lacks (Me)GlcA; GlcAT activity in microsomes of transiently GUX2 overexpressing N. benthamiana.	Rennie et al., 2012; Mortimer et al., 2010
GUX4	At1g54940	glucuronic A side chain?	GlcAT activity in microsomes of transiently GUX4 overexpressing N. benthamiana.	Rennie et al., 2012
GXM1	At1g09610	glucuronic A side chain methylation	Reduced MeGlcA (unchanged GlcA), glucuronoxylan methyltransferase activity after heterologous expression	Lee et al., 2012a
GXM2	At4g09990	glucuronic A side chain methylation	reduced MeGlcA (unchanged GlcA), glucuronoxylan methyltransferase activity after heterologous expression	Lee et al., 2012a
GXM3/ GXMT1	At1g33800	glucuronic A side chain methylation	reduced MeGlcA (unchanged GlcA), glucuronoxylan methyltransferase activity after heterologous expression	Lee et al., 2012a; Urbanowicz et al., 2012
RWA1	AT5G46340	Acetylation in secondary walls	Triple and quadruple mutant has reduced xylan acetylation	Lee et al., 2011b; Manabe et al., 2011; Manabe et al. 2013
RWA2	AT3G06550	Acetylation of XG and pectin		
RWA3	AT2G34410	Acetylation in secondary walls		
RWA4	At1g29890	Acetylation in secondary walls		
TBL29, eskimo1	At3g55990	Xylan acetyl transferase	Mutant has reduced xylan acetylation	Xiong et al., 2013

IRX15—involved in backbone elongation or transport?

Another gene pair suspected to be involved in xylan backbone biosynthesis is *IRX15/IRX15-L*. They contain the DUF579 domain and are expressed in high xylan containing *Psyllium* seeds and in *Arabidopsis* during secondary cell wall formation (Brown *et al.*, 2011; Jensen *et al.*, 2011). IRX15 localizes to the Golgi and an additional unidentified intracellular compartment (Brown *et al.*, 2011). Phenotypes in *Arabidopsis* mutants are irregular secondary cell wall deposition, increased methylation and a reduced degree of xylan backbone polymerisation. A conserved region for metal coordination like in the DUF579 family protein GXMT1—which is a methyltransferase—could suggest methyltransferase activity, but this is disproven by the highly methylated mutants (Urbanowicz *et al.*, 2012). The reduced degree of xylan backbone polymerisation rather suggests an involvement in backbone elongation, maybe as an important structural component of a protein complex. Alternatively, IRX15 could play a role in endomembrane transport, which is supported by the irregular cell wall deposition.

Evidence for a xylan biosynthetic complex

Why are several non-redundant enzymes required to perform the same biochemical function? One explanation could originate from xylan structure. To synthesise the β-(1-4)-linked xylan backbone, each xylosyl residue needs to be flipped around 180° relative to the next residue. This might require two glycosyltransferases—for example IRX9 and IRX14—that cooperatively add xylosyl sugars from opposite sides (Lee *et al.*, 2010).

Proof off a protein complex of glucuronoarabinoxylan biosynthetic proteins was experimentally given in wheat (Zeng *et al.*, 2010). Zeng (2010) demonstrated interaction between GT75, GT47 and GT43 family members using Co-IP and cooperative activity of xylan backbone synthesis and side chain addition which suggests a protein complex of the involved enzymes. However, 'Glucuronic Acid Substitution of Xylan 1' (GUX1) protein from *Arabidopsis* is able to add glucuronic acid residues to xylan substrate on its own (Rennie *et al.*, 2012). Nevertheless, despite the ability of an isolated protein to perform a specific enzymatic activity (to some degree) on its own, this does not exclude that it is a natural part of a protein complex. Such a protein complex can further enhance, control and coordinate specific enzymatic activities (Oikawa *et al.*, 2013).

The first biochemical evidence for a cooperative activity of *Arabidopsis* IRX9 and IRX14 comes from experiments where the genes were expressed individually as well as simultaneously in tobacco cells followed by

measurement of microsomal xylosyltransferase activity (Lee *et al.*, 2012b). Individual expression did not lead to measurable xylosyltransferase activity, whereas IRX9 and IRX14 cooperatively were able to incorporate xylosyl residues onto a xylotetraose (Xyl_4) acceptor. Xylooligomer substrates ranging from Xyl_3 to Xyl_6 were used to incorporate up to five xylosyl residues. On the other hand, rice IRX9 alone increases xylan synthase activity when overexpressed in *Arabidopsis*. This suggests IRX9 to be a rate limiting component, but does not exclude a complex with other proteins like IRX14 for optimal performance (Chiniquy *et al.*, 2013). Taken together, these findings strongly support the existence of a xylan biosynthetic complex at least containing IRX9 and IRX14.

1.6.2 The reducing end oligosaccharide

Glucuronoxylans of dicots and conifers contain a distinct oligosaccharide at their reducing end (REO): β-Xyl-(1-3)-α-Rha-(1-2)-α-GalA-(1-4)-Xyl (Peña *et al.*, 2007). The three glycosyltransferases FRA8/IRX7, IRX8/GAUT12 and PARVUS/GATL1 are required for REO biosynthesis in *Arabidopsis* (Wu *et al.*, 2010; Brown *et al.*, 2007; Lee *et al.*, 2007b; Peña *et al.*, 2007; Persson *et al.*, 2007; Brown *et al.*, 2005; Zhong *et al.*, 2005; Lao *et al.*, 2003). Mutating these genes, results in a reduction in glucuronoxylan content and almost complete loss of the reducing end sequence. The total number of chains decreased while their heterodispersity (varying sizes) increased. For comparison, in *irx9* mutants—which lack the xylan backbone elongating IRX9 enzyme—the REO is more abundant, indicating shorter xylan chains.

FRA8 belongs to family GT47 with an inverting enzymatic mechanism. It could theoretically catalyse the β-linkage in β-Xyl-(1-3)-α-Rha using UDP-α-Xyl substrate or the α-linkage in α-Rha-(1-2)-α-GalA using UDP-β-Rha substrate (Peña *et al.*, 2007). A functional redundant paralog of FRA8 is F8H (Wu *et al.*, 2010; Lee *et al.*, 2009). It can complement the *fra8* mutant, and the double mutant shows a more severe defect regarding reduction in cell wall xylose and vessel deformation.

IRX8 and PARVUS are GT8 family members with a retaining mechanism. Using α-GalA as sugar donor, they could catalyse α-linkage formation in α-GalA-(1-4)-Xyl, which is the very last (or first) glycosidic linkage in xylan. As PARVUS localizes to the ER, unlike the other Golgi-located xylan biosynthetic enzymes, it has been suggested to be involved in initiation of the reducing end oligosaccharide in the ER (Lee *et al.*, 2007b).

Interestingly, mosses and grasses likely have orthologs of *FRA*, *IRX8* and *PARVUS*, but lack the reducing end oligosaccharide. This could implicate that

these orthologous genes are not directly involved in REO biosynthesis (Rennie & Scheller, 2014).

1.6.3 Glucuronic acid side chains

The glucuronic acid side chains are added by proteins called Glucuronic Acid Substitution of Xylan (GUX) (Bromley *et al.*, 2013; Rennie *et al.*, 2012; Mortimer *et al.*, 2010; Oikawa *et al.*, 2010). Their glucuronosyltransferase activity has been biochemically proven via transiently overexpressing GUX1, GUX2 and GUX4 in *N. benthamiana* (Rennie *et al.*, 2012). For GUX1, even *in vitro* activity has been shown. The *gux1/2* double mutant is depleted of glucuronosyltransferase activity and glucuronic side chains (Mortimer *et al.*, 2010).

Interestingly, it was found that GUX1 and GUX2 add glucuronosyl residues to distinct xylan domains (Bromley *et al.*, 2013). GUX1 preferably adds GlcA to evenly spaced xylosyl residues which mostly are eight or 10 residues apart, resulting in the most abundant so-called major xylan domain. GUX2 decorates the minor xylan domain more tightly with GlcA substitutes—usually five, six or seven residues apart—on evenly as well as oddly spaced backbone residues. These distinct xylan domains could play an important role in cell wall assembly. Xylan may form either a threefold or a flat twofold screw structure with each sugar residue placed at 120°C or 180°C, respectively, from the next residue (Bromley *et al.*, 2013). Threefold screws are recognized by some carbohydrate modules, but are not effective for interaction with the cellulose twofold screw structure (Szabo *et al.*, 2001). A twofold screw structure of the xylan major domain though, would create an uncharged surface for xylan-cellulose interaction and a charged surface exposing the GlcA side groups for interaction with other cell wall components (Bromley *et al.*, 2013; Reis & Vian, 2004).

1.6.4 Xylan methylation

Methylation of xylan glucuronic acid side groups requires Glucuronoxylan methyltransferase (GXM) 1, GXM2 and GXM3, which belong to the same DUF579-containing family as IRX15 (Lee *et al.*, 2012a; Urbanowicz *et al.*, 2012). *gxm* mutants have reduced MeGlcA levels—with the most severe phenotype in *gxm2/3* double mutants—but no effect on plant growth, xylan xylosyltransferase activity or GlcA content. The low methylation level is atypical compared to many other xylan mutant phenotypes, which often exhibit complete methylation of their GlcA side chains (Brown *et al.*, 2009; Wu *et al.*, 2009; Peña *et al.*, 2007; Persson *et al.*, 2007; Zhong *et al.*, 2005). Lee (2012) found no change in the saccharification efficiency in g*xm* mutants. However,

Urbanowicz (2012) found increased xylan accessibility during enzymatic saccharification in mutated *gxm3* mutants (*gxmt1*). They concluded that the molecular interactions of cell wall polymers are altered due to the reduced methylation. The glucuronoxylan methyltransferase activity of GXM1, GXM2 and GXM3 was biochemically confirmed after heterologous expression in *E. coli* (Lee *et al.*, 2012a; Urbanowicz *et al.*, 2012). The recombinant proteins were able to transfer methyl groups on GlcA-substituted xylan acceptors, but not on GlcA monosugars, indicating that methylation *in vivo* takes place after addition of GlcA to the xylan chain.

GUX (glucuronosyltransferase) and GXM (glucuronoxylan methyltransferase) are the only purified xylan biosynthetic enzymes for which the putative activity has been biochemically characterised.

1.6.5 Xylan acetylation

Non-cellulosic cell wall polymers (pectin, lignin, xyloglucan, glucuronoxylan and glucomannan) are frequently O-acetylated; to a varying extent depending on species, tissue and cell wall type (Pawar *et al.*, 2013). Glucuoronoxylan is abundantly decorated with O-acetylgroups in the C-2 or C-3 positions of the backbone xylosyl residues. The total acetyl content in aspen wood is around 3.7% of the dry weight (Timell, 1967). The degree of acetylation (DA) describes the acetylation level; the ratio between total acetyl content and monomers that could be acetylated. Aspen xylan has a DA of 0.6-0.75 (Teleman *et al.*, 2002), which means that on average around 70% of the xylosyl residues contain an acetyl group.

Plant cell wall polymers are acetylated in the Golgi using acetyl-CoA as a donor substrate (Pauly & Scheller, 2000). In *Arabidopsis*, *REDUCED WALL ACETYLATION (RWA)* genes are required for cell wall polymer acetylation (Lee *et al.*, 2011b; Manabe *et al.*, 2011). RWA proteins contain the Cas1p domain with several transmembrane domains, which probably form a channel for acetyl-CoA transport from cytosol to Golgi lumen (Janbon *et al.*, 2001). Mutating all four *Arabidopsis RWA* genes reduces xylan acetylation by approximately 60% and negatively affects plant growth (Lee *et al.*, 2011b). RWA1, RWA3 and RWA4 proteins appear to be redundantly required for acetylation of xylan and other polymers, whereas RWA2 is more responsible for xyloglucan and pectin acetylation (Pawar *et al.*, 2013; Manabe *et al.*, 2011). Xylan acetylation in the Golgi lumen seems to be catalysed by TRICHOME BIREFRINGENCE-LIKE29 (TBL)/ ESKIMO1, containing the catalytic TBL and DUF231 domains (Xiong *et al.*, 2013; Anantharaman & Aravind, 2010). TBL-family members seem to be polymer specific acetyltransferases.

1.7 GT43 in *Populus*

The xylan backbone elongating *Arabidopsis* proteins IRX9 and IRX14 belong to glycosyltransferase family 43. GT43 members are present in the plant and animal kingdom, but not in primitive eukaryotes (Anders & Dupree, 2011). In plants, phylogenetic analysis divides the GT43 family into three clades, of which the IRX9/IRX9-L clades and the IRX14/IRX14-L clade are functionally non-redundantly required for xylan biosynthesis (Figure 7). The IRX9-clade seems to have evolved most recently in higher plants, as this is the only clade not represented in the non-vascular moss *Physcomitrella patens* and the vascular spikemoss *Selaginella moellendorffii* (Anders & Dupree, 2011). This implies a specialised function of the IRX9-clade in higher plants, which is supported by the severe xylem phenotype of *irx9* single mutants, compared to other GT43 family single mutants.

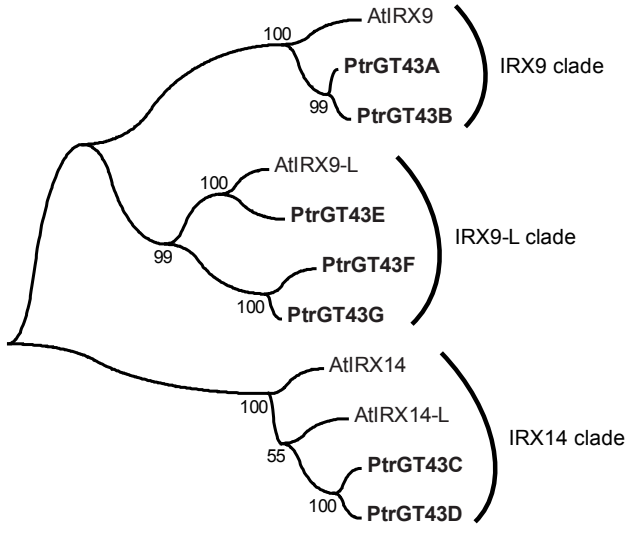

Figure 7. Phylogenetic relationship between *Populus trichocarpa* (Ptr) and *Arabidopsis thaliana* (At) GT43 family members. Neighbour-Joining tree with bootstrap values is based on Ptr and At GT43 protein sequences.

The *Populus* genome contains seven gene models predicted to encode members belonging to the carbohydrate active enzyme family GT43 (Geisler-Lee *et al.*, 2006; Tuskan *et al.*, 2006). Microarray-based expression profiling on developing xylem probes identified the two *IRX9* homologs, *GT43A* and *GT43B*, to be highly expressed in the secondary cell wall forming zone (www.popgenie.org) (Aspeborg *et al.*, 2005; Hertzberg *et al.*, 2001). *GT43E* was also found to be expressed in *Populus* tissues, but in contrast to *GT43A* and *B*, it exhibited a rather broad expression pattern (bar.utoronto.ca) (Wilkins *et al.*, 2009). *In situ* hybridisation experiments detected *GT43A*, *B*, *C*, *D* and *E*

mRNA in developing wood and their subcellular localisation appeared to be the Golgi in line with other xylan biosynthetic genes (Lee *et al.*, 2011a; Zhou *et al.*, 2007).

Overexpression of *Populus GT43* genes rescues *Arabidopsis irx9* and *irx14* mutant phenotypes, including vessel morphologies, xylan content and chain length as well as xylosyltransferase activities (Lee *et al.*, 2011a; Zhou *et al.*, 2007). The *irx9* mutant phenotype was functionally complemented by overexpressing *Populus* genes from IRX9 and IRX9-L clades (*GT43A*, *B* and *E*) but not by genes belonging to IRX14 clade (*GT43C* and *D*) and *vice versa*. This pattern of two functionally non-redundant groups within the *GT43* family, able to complement *irx9* or *irx14* mutant phenotypes, has also been shown for rice (Lee *et al.*, 2014). Those studies showed that *Populus* and rice *GT43* genes are functional orthologs of *Arabidopsis* genes and that the existence of two groups of functionally non-redundant xylan biosynthetic genes is conserved between annual and woody dicots as well as monocots.

A role of *Populus* GT43s in xylan backbone elongation was biochemically supported by co-expressing *Populus GT43B* (*IRX9* homolog) and *GT43C* (*IRX14* homolog) in tobacco (Lee *et al.*, 2012c). Tobacco microsomes co-expressing both genes exhibited successive xylan β-(1,4)-xylosyltransferase activity, in contrast to very little activity when individually expressed, indicating that GT43B and GT43C function cooperatively during xylan biosynthesis.

Table 3. *Populus* GT43 family members and evidence for their role in xylan biosynthesis.

***Populus* name**	***Populus* ID (v3)**	***Arabidopsis* ortholog**	**Evidence**	**Reference**
GT43A	Potri.006G131000	IRX9	Expression during scw formation Rescues *irx9 Arabidopsis* phenotype	Lee et al., 2011a; Aspeborg et al., 2005
GT43B	Potri.016G086400		Expression during scw formation; Rescues *irx9 Arabidopsis* phenotype; Cooperative XylT activity with GT43C in tobacco microsomes; RNAi in *Populus*: thin xylem cell walls, reduced xylan content and chain length	Lee et al., 2012c; Lee et al., 2011a; Zhou et al., 2007; Aspeborg et al., 2005; Hertzberg et al., 2001
GT43E	Potri.002G107300	IRX9L	Broad expression (Wilkins *et al.*) Expression during scw formation (Lee *et al.*) Rescues *irx9 Arabidopsis* phenotype	bar.utoronto.ca; (Wilkins et al., 2009); Lee et al., 2011a
GT43F	Potri.018G039900			
GT43G	Potri.006G240200			
GT43C	Potri.007G047500	IRX14/ IRX14-L	Expression during scw formation; Rescues *irx14 Arabidopsis* phenotype; Cooperative XylT activity with GT43C in tobacco microsomes	Lee et al., 2011a; Lee et al., 2012c
GT43D	Potri.005G141500	IRX14/ IRX14-L	Expression during scw formation Rescues *irx14 Arabidopsis* phenotype	Lee et al., 2011a

The role of GT43 genes during wood formation has been studied using an RNAi approach targeting *GT43B* for down-regulation in hybrid poplar (*Populus alba x tremula*) (Lee *et al.*, 2011a). Reducing *GT43B* expression levels led to thinner xylem fiber and vessel element cell walls with the typical collapsed vessel phenotype, reduction in xylan content and chain length, and to increased wood digestibility.

1.8 Biorefining Lignocellulose

"The Stone Age did not end for lack of stone, and the oil age will end long before the world runs out of oil."

Sheik Ahmed Zaki Yamani, 2nd Saudi Oil Minister (in office 1962-1986)

1.8.1 Biofuels from woody biomass

Plants are the source of many bio-based materials such as paper, chemicals, and liquid biofuels for human application. A biorefinery, in comparison to a conventional oil refinery, produces value-added products, such as fuels and chemicals from renewable raw materials. The advantage of biorefineries is the sustainability as well as the potential price of the raw material (Cavka, 2013).

Today, the most common biofuel is ethanol, made from starch- and sugar-based feedstocks like corn and sugarcane (Leboreiro & Hilaly, 2013). Their sugars are easy to extract and ferment and such feedstocks are therefore economically converted into fuels (U.S. Department of Energy, www.afdc.energy.gov). Lignocellulosic feedstocks are non-food feedstocks, such as dedicated energy crops, wood and agricultural residues and industrial wastes. They can be used for ethanol production, but this process is more challenging and not yet economically operable (Leboreiro & Hilaly, 2013). The advantages of lignocellulosic feedstocks over starch based feedstocks are their abundance, that they often are cheap waste products or grown specifically for bioenergy production and not used for human food (Keegstra, 2013). They can often grow on marginal land and are more economic and sustainable to grow.

There are two ways of producing ethanol from plant feedstocks: biochemical conversion and thermochemical conversion (U.S. Department of Energy, www.afdc.energy.gov). In biochemical conversion, a pretreatment (commonly acid) is applied to increase cellulose accessibility, followed by enzymatic saccharification to hydrolyse cellulose into soluble sugars, and finally microbial fermentation of sugars to ethanol (Figure 8) (Humbird *et al.*, 2011). During thermochemical conversion of plant material to ethanol—also called gasification—heat and chemicals are applied to produce syngas, a

mixture of carbon monoxide and hydrogen, which is then reformed into ethanol (Dutta *et al.*, 2011).

Lignocellulosic feedstocks contain cellulose, hemicelluloses and lignin to varying degrees, and this complex network of polymers is more recalcitrant to sugar release than starch- and sugar-based ethanol feedstocks. The level of recalcitrance has huge impacts on the biofuel processing efficiency and is highly dependent on the exact raw material composition—with woody biomass being especially challenging (Jönsson *et al.*, 2013).

Research to improve plant-based biofuel feedstocks targets yield and sustainability traits on one hand and processing traits on the other hand (Keegstra, 2013). Yield and sustainability traits regard general growth properties—the more yield the less space needed—as well as necessary irrigation, fertilisation and pest control. Processing traits are very specific and dependent on the technology used for further processing. Biochemical conversion of lignocellulose into ethanol, for instance, is highly dependent on the exact cell wall composition and its recalcitrance for optimal conversion efficiency; thermochemical conversion (gasification) on the other hand is less sensitive to cell wall composition.

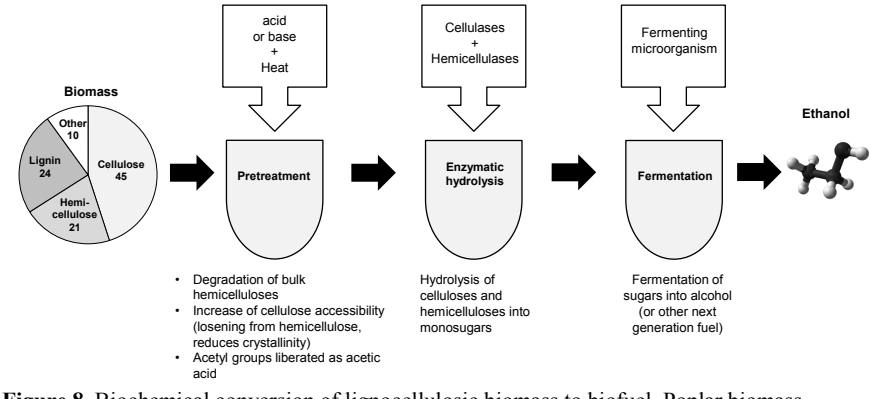

Figure 8. Biochemical conversion of lignocellulosic biomass to biofuel. Poplar biomass composition in % dry weight is indicated in the biomass chart (composition according to (Hu *et al.*, 2012)).

1.8.2 Cell wall network interactions

The recalcitrance of lignocellulose to degradation into monosugars is based on interactions between the different polymers cellulose, hemicelluloses and lignin. Cell wall polymers bond with covalent bonds, non-covalent hydrogen bonds, ionic bonds and van der Waals interaction between relatively unbranched polymer regions. Xylan decorations, such as glucuronic acids and acetyl groups, are likely to affect its conformation and solubility (Bromley *et*

al., 2013). Covalent bonds are thought to exist between hemicelluloses and lignin, resulting in lignin-carbohydrate complexes (LCCs) (Hu *et al.*, 2012). Lignin may be linked to GlcA side chains via ester bonds or to Xylose or Arabinose via ether bonds (Rennie & Scheller, 2014).

1.8.3 Xylan acetylation

Acetylation of glucuronoxylan inhibits interaction with polar, hydroxyl groups containing molecules, such as cellulose or water, as it reduces the available hydroxyl groups for hydrogen bonding (Pawar *et al.*, 2013). Thus, deacetylation promotes xylan-cellulose interaction as well as water absorption (Kabel *et al.*, 2007). This is important for biorefinery and pulping applications, as the deacetylated wood fibers swell and easily transport solutes (for example hydrolytic enzymes or chemicals). In pulping, the increased xylan-cellulose interaction after deacetylation increases yield and paper strength (Pawar *et al.*, 2013). For solid wood products on the other hand, acetylation is desirable as it increases mechanical strength.

During biofuel production, polysaccharides are hydrolysed—mostly enzymatically—to make the monosugars available for fermentation. Hemicellulosic acetyl groups sterically hinder enzyme accessibility, and deacetylation thus greatly improves saccharification efficiency (Biely, 2012). Further, high acetyl contents can negatively affect fermentation as the deacetylation generates acetic acid which inhibits yeast growth and fermentation (Pawar *et al.*, 2013).

Chemical deacetylation

Chemical pretreatment prior to enzymatic saccharification and fermentation in industrial applications usually uses acid, which hydrolyses the acetyl ester bond as well as the hemicellulosic glycosidic bonds, degrading the hemicellulose and leaving easily accessible cellulose (Jönsson *et al.*, 2013). Alkali treatment—which is used mostly in analytical applications—removes hemicellulosic acetyl groups and extracts the intact hemicellulosic polymers (Pawar *et al.*, 2013).

Enzymatic deacetylation

Enzymatic deacetylation can be performed together with polysaccharide hydrolysis during biofuel production. Xylan can be deacetylated by acetyl xylan esterases (AXEs) (EC 3.1.1.72), which are found in wood degrading fungi and bacteria, but not in plants (Biely, 2012). Acetyl xylan esterases belong to the CAZy carbohydrate esterase (CE) family (www.cazy.org).

To summarize, modification of cell wall acetylation levels can be beneficial for down-stream wood utilisation. Biofuel production gains from reduced polymer acetylation levels as this increases the accessibility off hydrolytic enzymes. Acetylation levels can be altered either via modification of plant endogenous acetylating genes or by introducing acetyl xylan esterases, but this requires more knowledge regarding the optimal degree and type of acetylation for plant performance as well as for down-stream application.

1.9 Xylan Modification

1.9.1 Proteins in the cell wall

The main cell wall structural components are cellulose, hemicellulose, pectin and lignin, which extensively interact via hydrogen bonds and covalent bonds. But likewise, proteins are important cell wall components, accounting for up to 10% of primary wall dry weight (Mellerowicz *et al.*, 2001). Cell wall proteins have either structural roles by being integrated components of the cell wall network, such as extensins and arabinogalactan proteins (AGPs), but can also have enzymatic functions (Kieliszewski *et al.*, 2011; Geisler-Lee *et al.*, 2006). Enzymatic cell wall modifications include, amongst others, hydrolysis of glycosidic and ester linkages as well as transglycosylation reactions (Figure 9) (Franková & Fry, 2013). Such cell wall polysaccharide remodelling is believed to represent a mechanism for controlled wall loosening and wall strengthening during growth, gravitropic responses and incorporation of new polysaccharide chains during wall deposition. Cell wall polysaccharide modifying enzymes belong primarily to the carbohydrate-active enzyme families of GHs, CEs and PLs (www.cazy.org).

An interesting example for a primary wall remodelling plant enzyme is XYLOGLUCAN ENDO-TRANSGLYCOSYLASE/HYDROLASE (XTH) (Eklöf & Brumer, 2010). XTH exhibits two distinct catalytic activities: xyloglucan endo-transglycosylase (XET) activity, which comprises the nonhydrolytic cleavage and ligation of two xyloglucan chains, as well as xyloglucan endo-hydrolase (XEH) activity, resulting in irreversible xyloglucan chain hydrolysis (Figure 9). Posing the question if other plant enzymes exhibit a similar dual hydrolytic and transglycosylase activity, Eklöf and Brumer (2010) reason, that the prerequisites—being a retaining endo-acting glycoside hydrolase—are fulfilled by the four GH families GH5 (mannanases, glucanases, galactanases), GH10 (xylanases), GH16 (xyloglucanases) and GH17 (glucanases). A mannanase with both hydrolytic and transglycosylating activity, LeMAN4a, was discovered and purified from tomato and suggested to be called MTH (mannan endotransglycosylase/hydrolases) in analogy to the

xyloglucan specific XTH (Schröder *et al.*, 2009). This MTH clearly exhibits dual enzymatic functions, but the respective biological significance is not yet clarified. Franková and Fry (2011) discovered in extracts of several plant species β-xylan endotransglycosylase activity which exceeded xylan hydrolase activity. Functional studies of those glycoside hydrolases will elucidate their polysaccharide remodelling activities and their effects on plant growth and properties.

Beyond characterised structural proteins and CAZymes, there are other cell wall proteins which appear essential for proper cell wall deposition, even though their exact roles are not understood. Examples are COBRA and COBRA-like genes, which are required for proper cellulose deposition, crystallisation and which affect cellulose microfibril angles (Liu *et al.*, 2013; Schindelman *et al.*, 2001).

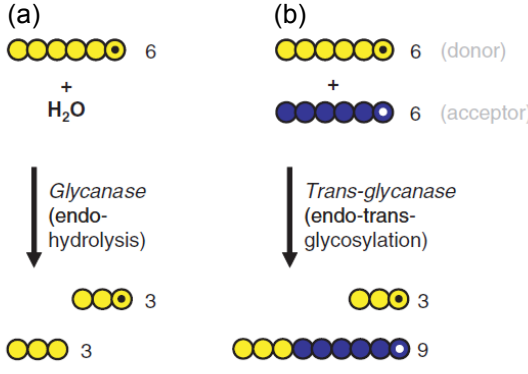

Figure 9. Polysaccharide hydrolysis and transglycosylation reaction schemes (Franková & Fry, 2011). Illustrated are reaction mechanisms of a glycanase (a), hydrolysing a hexaose molecule into two trioses; and a trans-glycanase (b), acting on two independent hexaose molecules (yellow and blue) producing a triose and a nonaose. Dotted circles represent orginial reducing ends.

1.9.2 Microbial xylan modifying enzymes

Besides plant endogenous CAZymes there exists a plethora of microbial lignocellulose degrading enzymes. Microbial enzymes for depolymerisation of the recalcitrant plant cell wall include glycoside hydrolases, pectate lyases and esterases (Gilbert, 2010; Gilbert *et al.*, 2008). Such microbial enzymes offer a great resource for targeted modification of cell wall polymers for a variety of down-stream wood utilisation.

Glucuronoxylan bonds can be hydrolysed by the following enzymes: carbohydrate esterases (CEs) hydrolyse as acetyl xylan esterases (AXE) the ester bonds of acetyl groups (CE1-7, CE12+13) (Pawar *et al.*, 2013) or as glucuronoyl esterases the ester linkages between glucuronic acid residues and lignin (CE15) (Li *et al.*, 2007; Špániková & Biely, 2006). Glycoside hydrolases (GHs) remove the glucuronic acid side groups (α-glucuronidase, GH67+GH115, (Gilbert, 2010) and degrade the xylan backbone (endo-1,4-β-

xylanases GH10 and GH11, and 1,4-β-xylosidase (GH3)) (Gilbert *et al.*, 2008; Goujon *et al.*, 2003). Of those xylan modifying enzymes, only xylanases and xylosidases were shown to exist in planta (Figure 10).

Xylan modifying enzymes can break lignin-carbohydrate cross-links and solubilize hemicelluloses, which finally increases carbohydrate accessibility for hydrolytic enzymes. Altering plant endogenous or expressing foreign microbial xylan active genes has potential to aid saccharification during biofuel production.

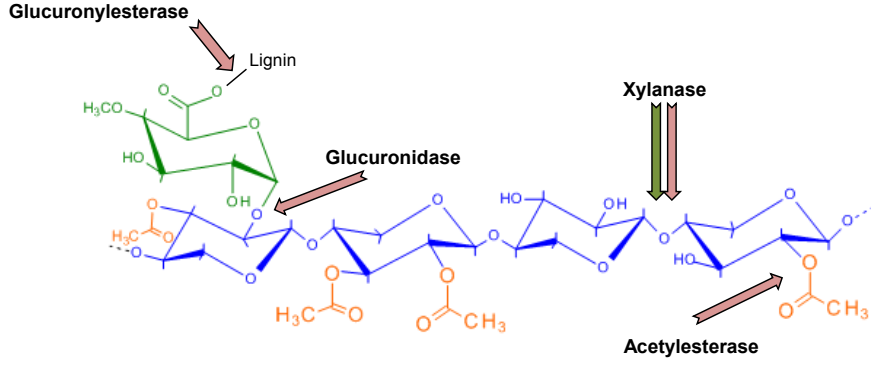

Figure 10. Xylan modifying enzymes. Indicated are plant endogenous (green arrow) as well as fungal (red arrows) hydrolytic activities on glucuronoxylan bonds.

1.9.3 Plant xylanases

Cell wall located xylan hydrolases in plants include exoxylanases (1,4-β-xylosidase (GH3)), which degrade xylan oligosaccharides from the nonreducing end, as well as endoxylanases (endo-1,4-β-xylanase (GH10), attacking the xylan chain at any position (Minic & Jouanin, 2006). Plant endogenous xylanases are known to have a degrading function in decomposing tissues, but their role during cell wall deposition is not understood (Simpson *et al.*, 2003).

Arabidopsis contains four expressed apoplastic endoxylanases belonging to the GH10 family (Suzuki *et al.*, 2002). One of these xylanases, AtXyn1 is expressed in vascular bundles and its overexpression increased xylanase activity. Thus, Xyn1 seems to have a role in secondary wall xylan metabolism, but its enzymatic properties and exact function remains to be characterised.

Despite the detection of xylan transglycosylase activity in plants, a corresponding enzyme has not been identified (Franková & Fry, 2011).

2 Objectives

The aim of this study was the characterisation of hybrid aspen (*Populus tremula* x *tremuloides*) genes involved in xylan biosynthesis and xylan modification. I studied these genes' physiological roles during cell wall formation, as well as their potential to improve wood properties for down-stream applications.

One aim of this study was the characterisation of the putative xylan backbone biosynthetic glycosyltransferase family 43 (GT43) in hybrid aspen. This characterisation included *GT43* family expression pattern analysis and targeted suppression to understand their biological importance during wood formation.

Subsequent xylan backbone biosynthesis, xylan chains can be modified by enzymes which, for example, hydrolyse the backbone: so-called xylanases. Determining the biochemical and physiological role during wood formation of a glycoside hydrolase with predicted xylanase function, was another aim of this study.

To study gene functions during secondary wall formation and to potentially modify wood properties using transgenic approaches, it is essential to efficiently target transgene expression to the tissue of interest. Therefore, another aim of this work was to identify and test a "wood-specific" promoter, which efficiently targets transgene expression at high levels specifically to secondary cell wall forming cells.

3 Results and Discussion

3.1 *Ptxt*Xyn10A is a Xylan Transglycosylase and Affects Cellulose Microfibril Angle (Paper I)

Plant tissues exhibit xylanase as well as xylan transglycosylase activities (Franková & Fry, 2013). Xylanases are known to be involved in tissue decomposition, for example during seed germination and fruit ripening (Simpson *et al.*, 2003). Their role during cell wall biosynthesis, where xylanases are also expressed, is not well understood (Geisler-Lee *et al.*, 2006; Suzuki *et al.*, 2002). Xylan transglycosylases are believed to play a rearranging role in the cell wall during polymer deposition, in analogy to xyloglucan endo-transglycosylase (XTH) (Eklöf & Brumer, 2010).

We studied the enzymatic mechanism of a xylan-active glycoside hydrolase during wood formation. *Populus trichocarpa* contains eight gene models encoding xylanases which belong to the CAZyme family glycoside hydrolase 10 (GH10), called *PtXyn10A* - *PtXyn10H* (Paper I, Figure S3) (Geisler-Lee *et al.*, 2006). Of these eight genes, only *PtxtXyn10A* was highly expressed in wood-forming tissues of hybrid aspen (*Populus tremula* x *tremuloides*) (Figure S4 + S5).

Xyn10A contains the catalytic GH10 domain and three carbohydrate binding modules (CBMs), theoretically resulting in a 101 kDa protein (Paper I, Figure S2). We identified a processing site predicting a 65 kDa mature peptide, which matched well with the protein we detected in xylem extracts (Paper I, Figure 1). The Xyn10A peptide accumulates in the cell wall, although it lacks a predicted signal peptide targeting it to the secretory pathway (Paper I Figure 1). Possibly, Xyn10A uses an unknown non-classical pathway to reach the apoplasm, which might require proteolytic processing (Agrawal *et al.*, 2010).

To assess which type of enzymatic activity is found in woody tissues, we measured xylanase and xylan transglycosylase activities in protein extracts

from developing hybrid aspen xylem (Paper I, Figure 2). Both activities were identified in a manner dependent on substrate-concentration. We found xylan transglycosylase activity when using high substrate concentrations and xylanase activity using low substrate concentrations. This is typical for dual hydrolase/transglycosylases, which use either water for irreversible hydrolysis or the carbohydrate substrate for transglycosylation reactions (Figure 9, Frankova 2014). The recombinant full-length hybrid aspen Xyn10A protein, expressed in *Arabidopsis* suspension cells, exhibits no significant xylanase activity but clear xylan transglycosylase activity (Paper I, Figure 3). Reducing Xyn10A protein level in transgenic hybrid aspen wood using an antisense approach, reduced xylan transglycosylase activity but not xylanase activity (Paper I, Figure 4). We conclude, that *Ptxt*Xyn10A from hybrid aspen is a xylan transglycosylase rather than a xylanase.

Studying the effects of reduced *Ptxt*Xyn10A levels on cell wall properties, we found no change in xylan chain length. This is consistent with an *in vivo* function as xylan transglycosylase and not as xylanase, as reduced xylanase levels would increase overall chain length. Instead, we found an altered cellulose microfibril angle (Paper I, Figure 7). The microfibrils were more axially oriented—they had a lower microfibril angle—in the *Xyn10A* antisense lines compared to the wild type. This suggests that cellulose microfibril angle orientation is influenced by xylan polymer modifications. One possible underlying mechanism could be that the glucuronoxylan coat confers negative charges to the cellulose microfibrils, which results in repulsive forces and thus altered microfibril interactions (Reis & Vian, 2004). Through which exact mechanism the glucuronoxylan-cellulose microfibril regulation takes place remains to be elucidated.

3.2 A *Populus* Glycosyltransferase Promoter Proves Useful for Targeted Wood Modification (Paper II)

Xylan backbone biosynthesis requires enzymes belonging to the GT family 43 (GT43). The poplar (*Populus trichocarpa*) genes *GT43A*, *B, C, D* and *E* were previously shown to be expressed in the developing xylem by *in situ* hybridisation (Lee *et al.*, 2011a). We measured the expression profiles of all seven hybrid aspen *GT43* family members in different plant tissues using quantitative RT-PCR (Paper II, Figure 1). *GT43A, B, C* and *D* expression was very high in the woody tissues compared to non-woody tissues (30 fold increase for *GT43A* and *B*), whereas *GT43E, F and G* exhibited a broad expression pattern. This result is in contrast to previous *in situ* results showing xylem specific expression of *GT43E* (Lee *et al.,* 2011a), but it is in agreement

with its microarray profile in *Populus* tissues (bar.utoronto.ca; Wilkins *et al.*, 2009) and the rather broad expression profile of its *Arabidopsis* ortholog *IRX9-L* (Wu *et al.*, 2010). We further confirmed the distinct expression pattern of *GT43E* using *GT43Epromoter::GUS* constructs, showing clear *GT43E* expression in primary cell walled phloem cells, but no expression in the xylem (Paper II, Figure 2). Altogether, the differing expression patterns could imply a diversification of GT43Es enzymatic role during xylan biosynthesis, for example with a specialised function in primary wall xylan formation.

One method to study the *in vivo* role of plant genes is to target the gene of interest for transcriptional gene silencing, for example using RNAi approaches. The right choice of promoter driving the transgene construct, with regard to strength of expression and cell specificity, is essential for the outcome of the experiment. We aimed at cloning a strong promoter which targets transgene expression specifically to secondary cell wall forming cells. The *GT43B* promoter seemed to be a good candidate based on the publicly available expression data including EST distribution, microarray profile and *in situ* hybridisation (Populus.db.umu.se; Lee *et al.*, 2011a; Aspeborg *et al.*, 2005; Sterky *et al.*, 2004). Our generated expression profiles using qRT-PCR, promoter::GUS analysis and MYB transactivation data substantiated the specific expression of *GT43B* in secondary wall forming tissues (Paper II, Figure 1-3). Therefore we constructed a plant expression vector containing the *GT43B* promoter, pK-pGT34B-GW, which is potentially useful for modifying transgene expression specifically during secondary cell wall formation (Paper II, Figure 4).

To assess the applicability of pK-pGT34B-GW for transgene expression in the wood, we compared its efficiency to the classical constitutive *CaMV 35S* (*35S*) promoter. For this purpose, we used each promoter to drive two transgenes with a quantifiable expected phenotype—to be specific, the level of cell wall acetylation—in stably transformed transgenic hybrid aspen. One transgene construct overexpressed a fungal carbohydrate esterase, which is known to remove xylan acetyl groups. The other construct was a down-regulating RNAi construct targeting the plant endogenous 'reduced wall acetylation' (RWA) family, which is required for unspecific cell wall polymer acetylation. Interestingly, the *pGT43B*-driven constructs were more effective than *35S* in reducing transgene levels and in achieving the intended chemical changes in the wood (Paper II, Figure 6+7). We conclude that the *GT43B* derived promoter has great applicability for targeting transgenes to secondary wall forming cells and to modify wood properties.

3.3 Application of the Wood Specific *GT43B* Promoter Enables Identification of a Sucrose Transporter in Developing Wood (Paper III)

Sugar precursors for cell wall biosynthesis are largely derived from sucrose, which is transported from photosynthesising source tissues through the phloem to the sink tissues. After long-distance phloem transport, sucrose enters the radial rays to finally reach the developing xylem cells. In *Populus*, uptake into these xylem cells seems to require plasma membrane-embedded sucrose transporters, as no plasmodesmatal connections between ray and vessel/fiber cells could be identified (Sokołowska & Zagórska-Marek, 2012). The aim of the paper III project was to identify the sucrose transporter responsible for sucrose import into developing xylem cells in *Populus*.

Of five sucrose transporter (*SUT*) candidate genes in the *Populus* genome, only *SUT3* is specifically expressed in the secondary cell wall forming tissue (Paper III, Figure 1). It is also highly expressed in the cambium, which is plausible considering the high energy demands during cell division as well as secondary cell wall formation. SUT3 has previously been shown to be a functional sucrose transporter in yeast uptake experiments (Payyavula *et al.*, 2011). Subcellular localisation studies using a *YFP:SUT3* fusion construct showed that the SUT3 protein is located in the plasma membrane, which supports its hypothesised role as a plasma membrane embedded sucrose importer (Paper III, Figure 2).

To analyse SUT3's *in vivo* role during secondary cell wall formation, we suppressed *SUT3* expression in transgenic hybrid aspen using an RNAi approach. In order to avoid a detrimental effect of cambial *SUT3* reduction on overall plant viability, *SUT3RNAi* construct expression was targeted specifically to secondary cell wall forming woody cells and not to cambial cells. To achieve such a 'wood-specific' expression we employed the *GT43B* promoter, which exhibits strong expression specific to secondary cell wall forming cells (Paper II). The RNAi mediated reduction of *SUT3* expression in developing wood was around 40% of wild type expression (Paper III, Figure 3), which is very similar to other experienced RNAi mediated gene silencing levels using the *GT43B* promoter (Paper II and IV). In the *SUT3RNAi* lines, plant growth was affected with a slight reduction in height and stem thickness and a 50% reduction in leaf size (Paper III, Figure 4). Xylem fiber walls were clearly thinner and the cellulose content per volume of wood was reduced compared to the wild type (Paper III, Figure 7). Thus, we concluded that the reduced *SUT3* RNA levels impaired carbon allocation to the developing wood.

To answer if the reduced cell wall biosynthesis was due to an overall reduction in carbon availability based on a reduced sucrose pool, we measured sucrose content in phloem and developing wood. However, there was no change in overall sucrose levels, but an increase in sucrose to hexose ratios which indicated a reduced conversion of sucrose into hexoses (Paper III, Figure 8). As the total wood sucrose pool did not appear to be limiting for cell wall polymer biosynthesis, we measured the carbon flux. $^{13}CO_2$ was supplied to two months old trees harbouring the *SUT3RNAi* construct, and ^{13}C accumulation was measured in the ethanol soluble fraction of developing wood (Paper III, Figure 9). *SUT3RNAi* lines accumulated more ^{13}C compared to the wild type, suggesting that the sucrose pool was not limiting, but instead the import into developing xylem cells was impaired.

Summing up, we found *SUT3*—a previously characterised functional sucrose transporter—to be plasma membrane localised and to be specifically expressed in *hybrid aspen* in carbon demanding cells during xylem formation: the cambium and the secondary cell wall forming zone. *SUT3*-RNAi mediated downregulation compromises secondary cell wall formation, which is not due to reduction of the total wood sucrose pool. Instead, *SUT3*RNAi lines accumulated ^{13}C in the ethanol soluble fraction, presumably due to reduced sucrose import into the growing cells, supporting a role of SUT3 as a sucrose importer in developing wood.

3.4 Reducing *GT43* Expression Levels in Hybrid Aspen Stimulates Cambial Proliferation and Improves Plant Growth, Wood Mechanical Properties and Saccharification (Paper IV)

To study the biological role of the xylan biosynthetic GT43 family and to investigate its potential in improving wood properties, we employed an RNAi approach to reduce *GT43* expression levels in hybrid aspen. To avoid functional compensation by paralogous genes, we constructed a set of specific RNAi constructs targeting either individual or multiple *GT43* clades for downregulation (Paper IV, Figure 1). Additionally, we used two different promoters, the constitutive *35S* and the wood-specific *GT43B* promoter (*pGT43B*) to control RNAi construct expression (Paper II + III). Utilisation of both promoters reduced xylem *GT43* expression levels comparably, to around 50% expression relative to WT (Paper IV Figure S1). However, overall growth was more affected in plants harbouring *pGT43B* than *35S*. We conclude that the two promoters differ in their cell-specificity, which is beneficial for targeted modification of secondary wall biosynthetic genes.

Interestingly, reducing *GT43* expression levels with the *GT43* promoter affected plant growth positively, contrasting with the dwarf phenotypes of corresponding *Arabidopsis* knock-out mutants. Most pronounced growth increase exhibited trees harbouring the RNAi construct targeting *IRX9* and *IRX14* clades for downregulation (*GT43BC*), confirming the essential role of these two functional non-redundant clades during wood formation (Paper IV, Figure 2). Subsequently, we focused on this *GT43*-RNAi construct (*GT43BC*) for more detailed phenotypic analysis.

Because GT43 members were shown to be involved in xylan biosynthesis, and *Arabidopsis* mutant phenotypes include reduction of xylan content and chain length, we characterised the cell wall composition and xylan structure (Paper IV, Figure 4). Cell wall composition was largely unaffected in the *GT43*-RNAi lines (*GT43BC*-RNAi construct), but we identified a modest reduction of xylan chain length. This finding substantiates a biological role of *Populus* IRX9 and IRX14 clade members (GT43A, B, C and D) during xylan backbone elongation (Paper IV, Figure 4).

With regard to potential applications as a bioenergy crop we were interested in the saccharification efficiency of *GT43*-RNAi lines. Interestingly, they yielded more monosugars after enzymatic saccharification, which could be explained with the reduced xylan chain length and its effects on cell wall network interactions. Studying the mechanical wood properties of dissected xylem specimen, we found increased mechanical strength in *GT43*-RNAi lines compared to wild type (Paper IV, Figure 5). The described increase in overall plant growth, mechanical strength and saccharification yield illustrates the enormous potential of xylan modification for customisation of wood for bioenergy and other uses.

To elucidate the underlying biological mechanism behind the increased plant growth in *GT43* RNAi lines, we first studied tissue and cell anatomy. Individual xylem fiber cells were only slightly bigger in the RNAi lines, and therefore an increased cell size (increased cell wall plasticity) could not explain the increase in tissue thickness (Paper IV, Figure 3). We concluded that enhanced cell division is the basis for the growth gain of transgenic trees with reduced *GT43* expression levels. To understand the underlying gene regulatory networks, we then studied the transcriptome using RNA sequencing. Strikingly, the whole secondary cell wall biosynthetic machinery was downregulated together with *GT43* genes, whereas regulatory factors involved in cambium maintenance and early xylem differentiation were upregulated (Paper IV, Figure 6). Such differential gene expression could stimulate cambial proliferation and explain the overall increase in biomass.

4 Conclusions and Future Perspectives

The aim of the first project was to understand xylan modification through plant endogenous glycoside hydrolases. In Paper I we identified a xylan transglycosylase in the wood of hybrid aspen. Previously, xylan transglycosylase activity—the nonhydrolytic cleavage and ligation of xylan chains—had been detected in plant tissues. The glycoside hydrolase family 10 member *Ptxt*Xyn10A was believed to be a xylanase, hydrolysing xylan chains, and its role during secondary cell wall deposition was unknown. Our enzymatic assays showed that *Ptxt*Xyn10A has transglycosylase activity, rather than xylanase activity, and that it is active during wood formation in cells depositing secondary walls. Additionally, we found that this xylan transglycosylase affected cellulose microfibril angles, which gives new insights into the interaction of cell wall polymers and their control during deposition into the wall.

To functionally study genes involved in secondary wall formation, a strategy is to modify gene expression in transgenic hybrid aspen. This requires a promoter which efficiently targets gene expression to secondary wall forming cells. We isolated and tested a promoter, pGT43B, which proved to be very efficient in altering gene expression and in inducing the intended modifications in woody cells (Paper II). This wood specific promoter vector is an important contribution to the field of wood research, as it facilitates the strong and specific expression of a gene of interest in secondary cell wall forming cells. We applied this *GT43B* promoter successfully to modify sucrose transport (Paper III), xylan polymerisation (Paper IV) and xylan acetylation (Paper II) in hybrid aspen wood.

GT43 glycosyltransferases are known to be involved in xylan biosynthesis from *Arabidopsis* mutant experiments. When I started this project nothing was known about the role of glycosyltransferases in xylan biosynthesis during wood formation, except their peaking expression in developing wood (Geisler-

Lee *et al.* 2006). I contributed to the field by depicting the expression patterns of individual *GT43* family members in different *Populus* tissues (Paper II). Especially interesting was the finding that gene members belonging to the two clades IRX9 and IRX14 (*GT43A, B, C* and *D*) all were specifically expressed in woody tissues, whereas one *Populus* gene, *GT43E* (the IRX9-L ortholog), showed a broad expression pattern. This implies a functional diversification of *GT43* gene members, for example with distinct roles during primary and secondary cell wall formation. Identification and description of such divergent xylan biosynthetic protein complexes is an interesting open question waiting to be answered.

The aim of project IV (Paper IV) was to understand the biological role of *Populus GT43* genes in xylan biosynthesis and wood formation. Simultaneous suppression of the gene members *GT43A, B, C* and *D*—belonging to IRX9 and IRX14 clades—confirmed their role in xylan backbone elongation in the wood. We furthermore found that reduced *GT43* expression levels positively affected overall plant growth, wood mechanical strength and saccharification efficiency. Such improved wood properties comprise a potential as a bioenergy feedstock. Future field trials could show if the observed advantageous growth and wood properties remain when plants are confronted with natural biotic and abiotic stresses.

Aiming at clarifying the underlying biological mechanisms of the observed growth gain in *GT43* suppressed trees, we found that the growth was based on increased cambial proliferation. In the *GT43* suppressed trees we then identified that regulating factors known to be involved in vascular proliferation were upregulated—probably explaining the increased cell division—whereas the secondary wall biosynthetic machinery was suppressed. How can suppression of xylan biosynthetic genes negatively affect other wall biosynthetic genes and how does it positively affect cambial proliferation? At which level does such a sensing and signalling mechanism exist in the wood; at the RNA, protein or cell wall structure level? These open questions are challenging matters to investigate in the future.

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