Acetylation of Polysaccharides in Plant Cell Wall

with a Focus on Woody Species

Prashant Mohan-Anupama Pawar

Faculty of Forest Genetics
Department of Forest Genetics and Plant Physiology
Umeå

Cover: Cross section of green-house grown *Populus* stem with magnified xylem cells and cell wall structure showing acetylated (green) xylan (red) interacting with cellulose surface.
(Photo: Marta Derba-Maceluch)

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Abstract

Plant cell wall in woody tissues is a complex matrix, which consists of cellulose, matrix polysaccharides and lignin. The matrix polysaccharides are substituted with acetyl group that are hypothesised to play important roles in determining properties of these polysaccharides. The aim of this thesis was to understand the role of *O*-acetylation in plants and investigate possibilities for improvement of woody lignocellulose for biorefinery applications by reducing wood *O*-acetylation.

To alter acetylation specifically in woody tissues, a promoter from Glycosyl Transferase 43 family (GT43) in *Populus* was isolated that had a very specific expression in secondary cell wall forming cells. This xylem specific promoter (*pGT43B*) was more effective in modification of wood acetylation by overexpression and suppression strategies as compared to *35S* promoter (Paper I).

To reduce xylan acetylation using transgenic approach, acetyl xylan esterase from *Aspergillus niger, AnAXE1,* was targetted specifically to the cell wall in Arabidopsis (Paper II) and in *Populus* (Paper III). Plants expressing *AnAXE1* grew as well as wild type and had increased acetyl esterase activity. This has led to reduction in cell wall acetyl content and in xylan *O*-acetylation. Moreover, transgenic Arabidopsis exhibited increased resistance against a biotrophic pathogen *Hyaloperonospora arabidopsidis.* Both transgenic plants had improved sugar yields in saccharification with different pretreatments and without pretreatment.

To reduce acetylation using cisgenic approach, *Populus* Reduced Wall Acetylation (RWA) gene family was characterised by suppression of the two clades RWA-AB, and RWA-CD (Paper IV). Both clades were shown to be involved in xylan acetylation in the wood. Both clades were therefore suppressed under control of xylem specific promoter *pGT43B* to improve wood saccharification potential. Transgenic plants had reduced wood acetyl content, normal growth, and increased sugar yield and glucose conversion % in saccharification without pretreatment. Glucose yield was also slightly increased in saccharification after acid pretreatment.

These results show that reduction of cell wall acetylation by 10-30% does not alter plant growth and development, but improves yields in lignocellulose saccharification (with and without pretreatment) (Papers II, III and IV).

To identify Quantitative Trait Loci (QTLs) related to cell wall acetyl content and other chemical traits in *Salix*, the mapping population of 463 progenies of *S. viminalis* and *S. schwerinii* was analysed by FT-IR and acetyl content assay (Paper V). 28 QTLs were identified for different cell wall chemical traits, which were co-located with several cell wall related genes and gene clusters. These QTLs and genes can be used in the future to improve wood chemical traits in *Salix* and *Populus* for biofuel production by breeding.

Keywords: acetylation, QTL, saccharification, secondary cell wall, xylan, Arabidopsis, aspen, *Salix*, RWA, acetyl xylan esterase

Author's address: Prashant Pawar, SLU, Department of Forest Genetics and Plant Physiology, SE-901-83, Umeå, Sweden
E-mail: Prashant.Pawar@slu.se

Dedication

To my family, teachers and research supervisors

"Make things as simple as possible, but not simpler" – Albert Einstein

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 Christine Ratke, **Prashant Mohan-Anupama Pawar**, Vimal K. Balasubramanian, Marcel Naumann, Mathilda Lönnäs Duncranz, Marta Derba-Maceluch, András Gorzsás, Satoshi Endo, Ines Ezcurra and Ewa J. Mellerowicz (2015). *Populus* GT43 family members group into distinct sets required for primary and secondary wall xylan biosynthesis and include useful promoters for wood modification. Plant Biotechnology Journal 13, 26-37.

II **Prashant Mohan-Anupama Pawar**, Marta Derba-Maceluch, Sun-Li Chong, Leonardo D. Gómez, Eva Miedes, Alicja Banasiak, Christine Ratke, Cyril Gaertner, Grégory Mouille, Simon J. McQueen-Mason, Antonio Molina, Anita Sellstedt, Maija Tenkanen and Ewa J. Mellerowicz (2015). Expression of fungal acetyl xylan esterase in *Arabidopsis thaliana* improves saccharification of stem lignocellulose. Plant Biotechnology Journal *in press.*

III **Prashant Mohan-Anupama Pawar**, Marta Derba-Maceluch, Sun-Li Chong, Madhavi Latha Gandla, Shamrat Shafiul Bashar, Tobias Sparrman, Patrik Ahvenainen, Mattias Hedenström, Ritva Serimaa, Martin Lawoko, Maija Tenkanen, Leif J. Jönsson and Ewa J. Mellerowicz. Expression of xylan acetyl esterase AnAXE1 improves saccharification potential and lignin solubility in aspen (Manuscript).

IV **Prashant Mohan-Anupama Pawar**, Christine Ratke, Vimal K. Balasubramanian, Sun-Li Chong, Madhavi Latha Gandla, Tobias Sparrman, Mathilda Lönnäs Duncranz, Ines Ezcurra, Maija Tenkanen, Leif J. Jönsson and Ewa J. Mellerowicz. Downregulation of RWA genes in hybrid aspen

affects xylan acetylation and improves wood saccharification properties. (Manuscript).

V **Prashant Mohan-Anupama Pawar**, Anna Schnürer, Ewa J. Mellerowicz, Ann Christin Rönnberg-Wästljung. QTL mapping of wood FT-IR chemotypes and acetyl content shows promise for improving biofuel potential in short rotation coppice willow (*Salix spp.*) (Manuscript).

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Additional publication and patent from the author which are not part of thesis

- **Prashant Mohan-Anupama Pawar**, Sanna Koutaniemi, Maija Tenkanen, Ewa J. Mellerowicz. Acetylation of woody lignocellulose: significance and regulation (2013). Frontiers in Plant Science. 4, 118.

- Yuzuki Manabe, Yves Verhertbruggen, Sascha Gille, Jesper Harholt, Sun-Li Chong, **Prashant Mohan-Anupama Pawar**, Ewa J. Mellerowicz, Maija Tenkanen, Kun Cheng, Markus Pauly, Henrik Vibe Scheller (2013). RWA proteins play vital and distinct roles in cell wall *O*-acetylation in *Arabidopsis thaliana*. Plant Physiology 163 (3), 1107-1117.

- **Prashant Mohan-Anupama Pawar**, Marta Derba-Maceluch, Ewa J. Mellerowicz, Madhavi Latha Gandla, Leif J. Jönsson. "Genetically modified plants having improved sachharification properties" International Publication Number WO 2013/137810 A1.

The contribution of Prashant Mohan-Anupama Pawar to the papers included in this thesis was as follows:

I   Experimental work and data analysis for cloning of CE5 construct, CE5 and RWA expression, FT-IR and acetyl content measurement. Preparation of the corresponding figures and parts of manuscript, reading and discussing the entire manuscript.

II   Experimental work, planning and data analysis for majority of experiments. Major part in writing the entire manuscript.

III Experimental work, planning and data analysis for majority of experiments. Major part in writing the entire manuscript.

IV   Experimental work, planning and data analysis for majority of experiments. Major part in writing the entire manuscript.

V   Supervision of FT-IR experiment, experimental work and data analysis for acetic acid analysis and data analysis for FT-IR. Preparation of corresponding figures/tables, and parts of manuscript, reading the manuscript.

Abbreviations

ACL	ATP Citrate Lyase
AGP	ArabinoGalactanProtein
ANOVA	ANalysis Of Variance
Ara	ARAbinose
AXE	Acetyl Xylan Esterase
AXY9	Altered XYloglucan 9
CE	Carbohydrate Esterase
DAMPs	Damage-Associated Molecular Pattern moleculeS
DP	Degree of Polymerisation
DUF	Domain of Unknown Function
Fuc	FUCose
Gal	GALactose
GalA	GALacturonic Acid
GDS	Gly-Asp-Ser
GH	Glycosyl Hydrolases
GHG	Green House Gases
Glc	GLuCose
GlcA	GLucuroniC Acid
GRPs	Glycine-Rich proteins
GUX	GlucUronic acid substitution of Xylan
GXM	GlucuronoXyloMannan
HG	HomoGalacturonan
HRGPs	Hydroxyl Proline-Rich Glycoproteins
MBOAT	Membrane-Bound O-Acetyl Transferase
meGlcA	MEthyl GLucuroniC Acid
*O*Ac	*O*-Acetylation
OS	OligoSaccharides
PAE	Pectin Acetyl Esterases
Pat A	Peptidoglycan *O*-AcetylTransferases A
PG	Peptidoglycans

PRPs	Proline-Rich Proteins
QTL	Quantitatve Trait Loci
RGAE	RhamnoGalacturonan Acetyl Esterase
RG-I	RhamnoGalacturonan I
RG-II	RhamnoGalacturonan II
Rha	RHAmnose
RWA	Reduced Wall Acetylation
SGNH	Serine-Glycine-Aspargine-Histidine
SNP	Single Nucleotide Polymorphism
TBL	Trichome Birefringence-Like
XG	XyloGlucan
Xyl	XYLose
Xyl*p*	XyloPyranose

Introduction

1.1   Why to study plant cell wall

According to recent report released by United Nations, the global population will not stabilise until the end of 21st century. It will go on increasing from current 7.2 billion to 12.3 billion in 2100 (Gerland *et al.*, 2014). To meet the food and energy demand for such a huge population, we need to find new food and energy resources and modify existing ones. Plants are serving this purpose since beginning of human race. Also, plant biomass which is mainly composed of "plant cell wall" can be used as an energy source in the form of heat as a raw material in different industries like paper, pulp, and textile (Pauly & Keegstra, 2008; Fenning & Gershenzon, 2002). Another important use of biomass based on plant cell wall is the production of biofuel. Using biofuels will exploit existing plant biomass and CO_2 released during processing of biomass will be consumed by plants for photosynthesis and the overall effect of CO_2 would be the same. Thus, this process will be carbon neutral. Moreover, it will also reduce the released CO_2 from fossil fuels. But, the intricate structure of plant cell wall is a major hurdle in processing of plant biomass for the efficient biofuel production. To meet growing energy demand and to avoid emission of Green House Gases (GHG) from fossils fuels, it is necessary to understand structure of plant cell wall and exploit it more efficiently for energy production.

1.2   Plant cell wall architecture

Plant cell is composed of different cell organelles and surrounded by a cell wall. Plant cell wall is important for overall plant growth and development. It helps in determining the cell´s growth, its final shape, and volume. It acts as a physical barrier which protects the plant from different biotic and abiotic stresses (Albersheim, 2011). Plant cell wall of xylem cells comprises primary and secondary cell wall layers that are deposited successively (**Figure 1**) (Mellerowicz & Gorshkova, 2012). Different types of xylem cells perform

different functions. Xylem vessel elements are involved in water transport; xylem parenchymatous cells are responsible for transport of solutes and thick-walled xylem fibers give mechanical support to the plant. Cells having primary cell wall layer only are capable of growth; this kind of cell wall is made up of mainly polysaccharides and glycoproteins. Secondary cell wall layer is deposited when the cell's growth has stopped and it consists of three cell wall layers which become impregnated with polyphenols after deposition of carbohydrate and protein components.
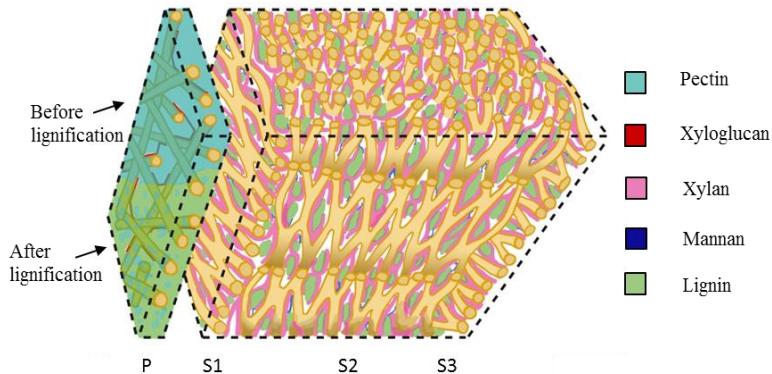
Figure 1. Plant cell wall model showing primary (P1) and Secondary (S1, S2 and S3) layers, different cell wall components in those layers (adapted from (Mellerowicz & Gorshkova, 2012)

1.2.1 Cellulose

Cellulose is a homopolymer found in all plants in both primary and secondary cell wall layers. It is composed of linear β, 1-4 linked glucose (Glc) units which form a glucan chain. Approximately 20 glucan chains are held together to form cellulose microfibril with 3-5 nm diameter size (Fernandes *et al.*, 2011). These cellulose microfibrils are a major load bearing structure which provides strength and flexibility to the plant cell wall. The percentage of cellulose is different in different plants and tissue types, and ranges from 20% to 95%. (Albersheim, 2011; Brown, 2004; Gardner & Blackwel.J, 1974).

1.2.2 Xyloglucan

Xyloglucan (XG) is predominantly found in primary cell wall layers of all higher plants. XG is a branched polymer and its backbone is made up of β, 1-4 Glc units. In most the plants, Carbon-6 (C-6) of Glc unit is substituted with xylose (Xyl) and Xyl can be further linked to galactose (Gal), fucose (Fuc), galactose (Gal), arabinose (Ara) depending on the species. Different domains of XG are tightly bound to cellulose by non-covalent interactions, which

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strengthen the structure of primary cell wall (Pauly *et al.*, 1999; Hayashi, 1989). The amount of XG, its substitution pattern and deposition in tissue types varies between dicots and grasses.

1.2.3 Pectin

Pectins are complex polysaccharides mainly present in primary cell wall layers. The percentage of pectin varies between grasses and dicots. Depending on their structure, pectins are divided into homogalacturonan (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II). HG is a homopolymer and its backbone is composed of α, 1-4 linked galacturonic acid (GalA). HG is the most abundant pectin and its content may reach 65% of cell wall dry weight (Albersheim, 2011; Mohnen, 2008). RG-I has α, 1-4 linked GalA and Rha disaccharide backbone and the rhamnose can be substituted with Ara, Gal, Fuc and glucuronic acid (GlcA) residues. Typically, in dicots 20 - 35% of pectins are RG-I. RG-II has the same backbone as HG but two types of oligosaccharide chains with different sugar composition are attached to C-2/C-3 carbons.

1.2.4 Cell wall proteins

These are primarily present in primary cell wall layer matrix and account for 1-10% of dry weight of this cell wall layer. They are divided into two groups based on their solubility. The insoluble proteins include hydroxyl proline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs) and proline-rich proteins (PRPs). The soluble proteins are present in the form of enzymes, lectins and arabinogalactan proteins (AGPs). 10% of cell wall dry weight consists of AGPs and 95% of their weight is glycosylate decorations (Albersheim, 2011). In Arabidopsis cell wall, AGPs is covalently attached to RG-I through rhamnosyl residue of arabinogalactan (AG). Also, AGP can directly bind to arabionosyl residue of arabinoxylan (Tan *et al.*, 2013).

1.2.5 Xylan

Xylan is predominantly found in primary and secondary wall layers of grasses and in secondary wall layers of dicotyledons. It is made up of β, 1-4 linked Xyl units. Xylan from grasses is substituted with Ara, GlcA and methyl glucuronic acid (meGlcA) whereas in dicots secondary wall layers mostly GlcA and meGlcA substitutions are seen. The substitution of Ara, GlcA and meGlcA varies among different species (Ebringerova & Heinze, 2000). Arabidopsis xylan has two domains depending on spacing of GlcA/meGlcA (Bromley *et al.*, 2013) and molecular dynamics stimulation showed hydrophilic and hydrophobic interactions between xylan and cellulose (Busse-Wicher *et al.*, 2014).

1.2.6 Mannan

Mannan is a polysaccharide which is mainly found in secondary cell wall layers. Hardwood mannan is heteropolymer comprising of mannose (Man) and Glc units which are linked with each other by β, 1-4 glycosidic bonds. Softwood mannan has same backbone as hardwood but C-6 Man is substituted with Gal. The proportion of Glc and Man in mannan differs between hardwood and softwood species (Fengel & Wegener, 1984).

1.2.7 Lignin

Lignin is a heteropolymer which is formed after oxidative coupling of different monomers of hydroxyl cinnamyl alcohols. Lignin is mainly found in cells forming secondary cell wall layers and it is also deposited in other cell types in response to different biotic and abiotic stresses. Lignins are formed from three types of hydroxyl cinnamyl alcohols - *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol which are respectively incorporated into lignin polymer in the form of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monolignol units (Vanholme *et al.*, 2010). H lignin is present in minor quantity mainly in grasses and in gymnosperms. G lignin is the main lignin component in gymnosperms. Angiosperm lignin is composed of S and G units.

1.3   What makes plant cell wall recalcitrant?

Cell wall is complex matrix and properties of individual polymers and cross linking between them, make it resistant to enzymatic degradation during biofuel production.

Cellulose microfibrils containing 500-14000 Glc units are tightly arranged to form crystalline structures 200-300 nm long separated by amorphous regions (Zhao *et al.*, 2012). Amorphous region is 3-30 times easier to hydrolyse than the crystalline part as tight hydrogen bond arrangement pattern makes it difficult for hydrolytic enzymes to penetrate in crystalline region (Zhu *et al.*, 2008). It is also argued that crystallinity of cellulose does not have impact on enzymatic hydrolysis but rather a high degree of polymerisation (DP) of cellulose makes it slow (Zhao *et al.*, 2012; Puri, 1984). Cellulose is a long homopolymer and requires endo- and exo- glucanases and β-glucosidases (Horn *et al.*, 2012) to convert it to Glc, and since the process is long; accumulating hydrolysis products can inhibit enzymes activity. For example, cellobiose is known to inhibit activity of cellulases (Duff & Murray, 1996). Cellulose is also attached non-covalently to XG, xylan and pectin by hydrogen bonding. These interactions might have direct effect while processing biomass (Busse-Wicher *et al.*, 2014; Hayashi, 1989). To disrupt the structure of cellulose many approaches have been tried. Expression of Glycosyl Hydrolases (GH) *in planta* has been one of the approaches to make it less recalcitrant

(Klose *et al.*, 2015; Garvey *et al.*, 2013; Brunecky *et al.*, 2011). Accumulation of cellulases in different cellular compartments has been achieved in above studies. This increases cellulase yield in plants which increases digestibility of cellulose during saccharification. But, the growth in such transgenic plants has been hampered along with negative change in cell wall properties and stress tolerance (Petersen & Bock, 2011; Taylor *et al.*, 2008; Abdeev *et al.*, 2004). Overexpression of *KORRIGAN* has been successful in reducing crystallinity of cellulose (Maloney & Mansfield, 2010; Takahashi *et al.*, 2009) but in some cases it has led to impairment in growth of plant and deformed xylem cells.

Lignin is another important factor which increases biomass recalcitrance. It is dispersed as a cementing material in cell wall. It is well known that lignin abundance is negatively correlated with biomass digestibility (Zhu *et al.*, 2008; Holtzapple & Chang, 2000). Its close association with cellulose inhibits biomass digestibility. Apart from blocking way of cellulases, it also absorbs cellulases on its surface which restrict cellulose digestibility (Zhao *et al.*, 2012; Mansfield *et al.*, 1999). Different pretreatments are used to at least partially remove lignin prior to enzymatic carbohydrate hydrolysis. Also, to reduce the lignin content in plant biomass, genes involved in lignin biosynthetic pathway have been engineered by different transgenic approaches. Antisense transgenic alfalfa plants having downregulation in different lignin biosynthetic genes were tested for enzymatic saccharification after acid pretreatment. Glc and Xyl release was increased in many of these transgenic lines. Negative correlation was seen between lignin content and sugar release. This suggests that decrease in lignin amount increases the accessibility for hydrolytic enzymes to cellulose and residual hemicellulose. Similar type of correlation was found in different plant species having modified lignin content and composition (Van Acker *et al.*, 2014; d'Yvoire *et al.*, 2013; Van Acker *et al.*, 2013; Cook *et al.*, 2012; Fornale *et al.*, 2012; Jung *et al.*, 2012; Mansfield *et al.*, 2012; Papa *et al.*, 2012; Fu *et al.*, 2011a; Fu *et al.*, 2011b; Studer *et al.*, 2011; Xu *et al.*, 2011; Dien *et al.*, 2009). However, transgenic plants with reduced lignin content usually have growth defect, increased susceptibility against pathogens resulting in decreased total biomass yield. (Reviewed in (Bonawitz & Chapple, 2013; Vanholme *et al.*, 2010). To avoid such scenario, there have been successful attempts to engineer the plants so that they form an artificial type of lignin which is easy to degrade. These transgenic plants do not show any growth defect (Bonawitz *et al.*, 2014; Wilkerson *et al.*, 2014).

Presence of hemicelluloses in cell wall makes interaction with cellulose and lignin tighter. Removal of hemicelluloses by dilute acid pretreatment, or by heating to the temperature higher than $160°C$ increases digestibility of lignocellulose (Zhao *et al.*, 2012). Xylan which is the most of abundant hemicellulose in grasses and dicots can be removed by xylanases. If xylanases are used to treat lignocellulose, it leads to effective removal of lignin fragments

attached to xylan (Mansfield *et al.*, 1997). Expression of xyloglucanases in poplar made crystalline cellulose more susceptible for enzymatic degradation by loosening the XG structure (Kaida *et al.*, 2009). Also, overexpression of xylanases in plants improved Xyl and Glc yield in enzymatic saccharification and less exogenous enzymes was needed to hydrolyse the biomass (Zhang *et al.*, 2011a). Reduced xylan content was found in transgenic poplar having reduced expression of xylan biosynthetic genes and this has improved yield of sugar during saccharification (Lee *et al.*, 2011c; Lee *et al.*, 2009). However, the transgenic trees had irregular xylem phenotype, which probably would inhibit water transport and eventually hamper the growth. *xax1 (in Oryza sativa) and sac1* (in *Brachypodium distachyon*) mutants show improvement in saccharification and these mutants lack Ara side chains in xylan, and have decreased contents of ferulic acid, Xyl and *p*-coumaric acid (Marriott *et al.*, 2014; Chiniquy *et al.*, 2012). Unfortunately, *xax1* mutants have dwarf phenotype.

It has been also proposed that side groups/chains present on matrix polysaccharides are interacting with cellulose which could be one of the factors for biomass recalcitrance. Certain modifications in side chain/ groups of matrix polysaccharides increased digestibility of cellulose. It is shown that pectin methyl esterification positively regulates enzymatic saccharification of biomass. Improvement in enzymatic saccharification has been found by protecting methyl ester groups, or by reducing the content of HG. Overexpression of aspen pectate lyase has increased the solubility of pectin and hemicelluloses and also improved saccharification (Biswal *et al.*, 2014).

All matrix polysaccharides are *O*-acetylated. In wood, acetylation can be found from 1.5% to 4.4% (**Table 1**). 40-70% of Xyl*p* residues in hardwood xylan can be acetylated. Hydrolysing acetyl groups by acetyl xylan esterase *in vitro* increases accessibility for xylanases which will eventually help for digestibility of cellulose (Varnai *et al.*, 2014; Zhang *et al.*, 2011b; Selig *et al.*, 2009). Increase in Xyl and Glc yield was seen after chemical removal of acetylation from wheat straw and poplar wood (Kong *et al.*, 1992; Grohmann *et al.*, 1989). Bulkier nature of acetyl groups increases steric hindrance which makes difficult for hydrolytic enzymes to act on polysaccharides. Although, the effect of acetylation on lignocellulose digestibility was found to be minor compared to the impact of the crystallinity of cellulose and the high lignin content, it is still important factor for biomass recalcitrance (Zhu *et al.*, 2008). Furthermore, acetate is accumulated in the medium in the form of acetic acid after hydrolysis of lignocellulose. This changes pH of the medium, particular concentration of acetic acid might be inhibitory for microbes which are carrying out the fermentation process (Jonsson *et al.*, 2013; Helle *et al.*, 2003; Ranatunga *et al.*, 1997; Olsson & HahnHagerdal, 1996). To circumvent these negative effects, it has recently been proposed to use enzymes which convert

acetate to acetyl-CoA, then to acetyl aldehyde and to ethanol (Wei *et al.*, 2015). But, this study was done on isolated substrate and to bring it into practice for lignocellulosic biomass conversion requires to engineer the yeast strain, which is a major challenge. Moreover, use of chemicals and enzymes to deregulate the effect of acetylation on plant biomass is energy intensive, costly and environmentally hazardous process. According to hypothetical model, 20% reduction acetylation of biomass will decrease the cost of ethanol by 10% (Klein-Marcuschamer *et al.*, 2010). To exploit acetylation effectively and to improve biomass quality, it is important to understand role of acetylation in polysaccharides in plants, and to study biosynthesis of acetylation.

Table 1. Acetyl contents in some woody species in comparison with wheat straw (Pawar et al., 2013) (Reproduced with permission)

Species	% of d.w
Wheat	2.2
Populus tremuloides Michx.*(wood)*	3.7
Eucalyptus globulus Labill. *(wood)*	3.5
Fagus.grandifolia Ehrh. *(wood)*	3.9
Betula papyrifera Marsh. *(wood)*	4.4
Picea glauca Moench.*(wood)*	1.3
Pinus strobus L. *(wood)*	1.2
Abies balsamea Mill. *(wood)*	1.5

1.4   Acetylation in different cell wall polymers

All the cell wall polymers are *O*-acetylated except cellulose, mixed linked glucan and cell wall proteins (Pawar *et al.*, 2013; Gille, 2012). The degree and location of acetylation is different in polysaccharides and species. In addition to polysaccharides, lignin can also be *O*-acetylated (**Table 2**). Side chains of S and G lignin are *O*-acetylated at γ-carbon position in angiosperms but not in gymnosperms (Del Rio *et al.*, 2007). S lignin is highly acetylated in plants like abaca, sisal, kenaf and hornbeam up to 45% of S units. The substitution with acetyl groups will have impact on properties of polysaccharides and their interactions with other polysaccharides and other polymers in cell wall.

1.5   Biosynthesis of *O*-acetylation (*O*Ac) in cell wall polysaccharides

Biosynthesis of matrix polysaccharides is happening in the Golgi and many proteins involved in biosynthesis of *O*Ac of polysaccharides are known to localize in endoplasmic reticulum/Golgi membrane (Schultink *et al.*, 2015; Lee *et al.*, 2011a; Manabe *et al.*, 2011). Using microsomal preparation isolated

from potato cells, it has been shown that acetyl-CoA is a donor substrate for *O*Ac in XG and pectin (Pauly & Scheller, 2000).

Acetyl-CoA is an important metabolite present in plants, which is involved in different anabolic processes. It is produced in different cellular compartments like plastids, peroxisomes, nucleus and cytoplasm (Choudhary *et al.*, 2014; Fatland *et al.*, 2002). The precursors for acetyl-CoA and its function are different in different compartments. Moreover, it cannot diffuse through the membrane (Oliver *et al.*, 2009). The Golgi compartment does not have a possibility to synthesize the acetyl-CoA and it is hypothesized that the pool of acetyl-CoA in the Golgi, which is utilized for *O*Ac of cell wall polysaccharides is transported from the cytoplasm through a transporter (Gille, 2012).

Table 2. *O*-acetylated cell wall polysaccharides and lignin (Gille, 2012) (Reproduced with permission)

Wall Polymer	Species	Backbone/side chain	Residue	Position	Mono-/ Di-acetylated
XG	Dicots	Side chain	Gal	*O*-6; (*O*-3,*O*-4)	Mono/di
	Solanaceae	Backbone	Glc	*O*-6	Mono
		Side chain	Ara*f*	*O*-5	Mono
	Poaceae	Backbone	Glc	*O*-6	Mono
(Glucurono) arabinoxylan		Backbone	Xyl	*O*-2,*O*-3	Mono/di
		Side chain	Araf	*O*-2	Mono
Gluco(galacto-)mannan		Backbone	Man	*O*-2,*O*-3	
HG		Backbone	GalA	*O*-2,*O*-3	Mono/di
RG-I		Backbone	GalA	*O*-2,*O*-3	Mono/di
		Backbone	Rha	*O*-3	Mono
RG-II		Sidechain	2-*O*-Me-Fuc	n.d	Mono
Lignin			Syringyl-Guaucyl-units	γ-C of side chain	Mono

n.d = not determined

1.5.1 Metabolism of acetyl-CoA in the cytoplasm

Cytosolic acetyl-CoA can be utilized for acetylation, carboxylation and condensation processes (Oliver *et al.*, 2009; Fatland *et al.*, 2005). Acetyl-CoA cytosolic pool is responsible for acetylation of alkaloids, anthocyanins, isoprenoids, phenolics and sugars. Condensation of acetyl-CoA leads to formation of isoprenoids. Malonyl-CoA is formed after carboxylation of acetyl-CoA that helps in formation of elongated fatty acid like suberin, cutin and seed oil. Malonyl-CoA can also be source for formation of flavonoids.

Cytosolic acetyl-CoA is formed from citrate and CoA by ATP dependent conversion and this reaction is catalysed by ATP citrate lyase (ACL, (Fatland *et al.*, 2005)). ACL composed of two subunits (ACLA and ACLB) encoded by different genes. The antisense suppression of ACLA in Arabidopsis plants reduced the acetyl-CoA formation in cytosol. These plants showed defect in organ development, change in metabolic flux i.e. a reduction in cutin, and an increase in starch and anthocyanin, and an increase in stress related mRNA. Exogenous application of malonyl-CoA to antisense *ACLA* plants reverted severe plant phenotype to normal WT plants, which had normal anthocyanin production. This study suggests that the cytosolic acetyl-CoA pool impacts plant growth and development.

1.5.2 Biosynthesis of *O*-acetylated polysaccharides in the Golgi

O-acetylation biosynthetic machinery in the Golgi is found in fungi, bacteria, mammal and plants (Gille, 2012). *O*-acetylation mechanism shares some similarity in these systems. In *Cryptococcus neoformans*, glucuronoxylomannan (GXM) is *O*-acetylated in the capsule, which determines its properties (Janbon *et al.*, 2001; Cherniak & Sundstrom, 1994). Peptidoglycans (PG) from both Gram- positive and Gram-negative bacteria are *O*-acetylated, which protects PG from degradation by endogenous autolysins and lysozymes (Moynihan & Clarke, 2011). In human, sialic acid attached to reducing end of glycoproteins and glycolipids is also *O*-acetylated (Arming *et al.*, 2011). The first *O*-acetyl transferase was discovered in *C. neoformans* involved in acetylation GXM (Janbon *et al.*, 2001). The identified protein called *Cn*Cas1p protein has 12 transmembrane domains. The sequence orthologous to *Cn*Cas1p is identified in human (CasD1) and it is involved in acetylation of sialic acid (Arming *et al.*, 2011). These proteins have N-terminal serine-glycine-aspargine-histidine (SGNH) domain and multiple transmembrane domains at C-terminal. Mechanism of PG acetylation is different in Gram-positive than in Gram-negative bacteria. PG acetylation in Gram-positive bacteria is carried out by membrane-bound *O*-acetyltransferase (MBOAT) which has N-terminal acetyl transferase (SGNH) and C-terminal transmembrane domains (Moynihan & Clarke, 2011). On the other hand, acetylation of PG in Gram-negative bacteria needs two proteins, peptidoglycan *O*-acetyltransferases A (Pat A) and Pat B. Pat A is a member of MBOAT family and has a transmembrane domain and Pat B is *O*-acetyl transferase (Moynihan & Clarke, 2010). Pat A is probably involved in translocating acetylation from cytoplasm to periplasm and Pat B is transferring it to PG inside the periplasm. *O*-acetylations in *C. neoformans*, human and Gram-positive bacteria are carried out by single protein containing several transmembrane and one transferase domains. On the other hand, acetylation for

Gram-negative bacteria needs two separate proteins having two different domains (Gille, 2012).

Mechanism of *O*-acetylation of plant polysaccharides is similar to that of Gram-negative bacteria but it involves an additional protein component. Thus, in Arabidopsis, three gene families were proposed to be involved in *O*-acetylation of polysaccharides: reduced wall acetylation (*RWA*), trichome birefringence-like (*TBL*), and altered xyloglucan 9 (*AXY9*) (Schultink *et al.*, 2015; Gille, 2012). As discussed before, acetyl-CoA could be a donor substrate for acetylation but presence of another donor /intermediate substrate cannot be ruled out.

There are four RWA proteins in Arabidopsis, RWA1-RWA4 (Manabe *et al.*, 2011). These proteins have multiple transmembrane domains. *rwa2* Arabidopsis mutant showed defect in acetylation of xylan, pectin and XG (**Table 3**). Different combinations of double/triple mutants showed reduction in xylan and mannan acetylation suggesting, the RWA proteins might have redundant functions (Manabe *et al.*, 2013). A quadruple *rwa1/2/3/4* mutant doesn't show complete deacetylation in cell wall polysaccharides implying the possibility of other acetyl transporters (Manabe *et al.*, 2013; Lee *et al.*, 2011a). Plant RWA proteins probably have similar function to C-terminal transmembrane domains of *C. neoformans,* human and Gram positive bacteria and Pat A protein of Gram-negative bacteria.

Recently characterised *AXY9* gene family is plant specific (Schultink *et al.*, 2015). The *axy9* mutants exhibited reduction in xylan and XG *O*-acetylation but not in pectin *O*-acetylation (Table 3). The protease protection and split YFP assay have shown that AXY9 interacts with RWA and TBL proteins.

Arabidopsis *TBL* gene family has 46 members (Gille, 2012). According to protein structural analysis, TBL protein has one transmembrane domain, TBL domain (Gly-Asp-Ser (GDS)) and Domain of Unknown Function 231 (DUF231) (Bischoff *et al.*, 2010). TBL and DUF231 are found to be part of lipase esterase superfamily. It is hypothesised that one of the domains is involved in binding polymer and another one to substrate contributing for transferring acetyl group to polysaccharides (Gille, 2012). TBL and DUF231 also exist in human, Gram-positive, and Gram-negative bacteria. Mutation in *TBL27/AXY4* leads to complete XG deacetylation in different tissues except in the seed. Acetylation of XG in seeds is carried out by TBL22/AXY4L which is a paralog of AXY4. TBL22 and TBL27 are categorised as XG acetyl transferases (Gille *et al.*, 2011). Mutants in TBL29/ESK1 gene exhibited deacetylation of xylan and mannan (Xiong *et al.*, 2013; Yuan *et al.*, 2013). *In vitro* activity assay of TBL 29 protein showed that it is involved in transfer of acetyl group to xylan oligosaccharides but not to mannan oligosaccharides (Urbanowicz *et al.*, 2014).

Table 3. Mutants and transgenic lines with altered O-acetylation in cell wall polysaccharides in plants

Gene family	Mutant/OE	Defect in specific polysaccharide				References
		Pectin	XG	Xylan	Mannan	
RWA	*rwa2-3*	†	†	†	nr	(Manabe *et al.*, 2011)
	rwa2-1	†	†	†	nr	(Manabe *et al.*, 2011)
	rwa1/3/4	nr	†	†	†	(Manabe *et al.*, 2013)
	rwa2/3/4	nr	†	†	†	(Manabe *et al.*, 2013)
	rwa1/2/4	nr	†	†	†	(Manabe *et al.*, 2013)
	rwa1/2/3	nr	†	†	†	(Manabe *et al.*, 2013)
	rwa 1/2/3/4	nr	nr	†	nr	(Manabe *et al.*, 2013)
	rwa 1/2/3/4	nr	nr	†	nr	(Lee *et al.*, 2011a)
TBL	*axy4 (tbl27)*	⁻	†	⁻	nr	(Gille *et al.*, 2011)
	axy4L-2 (tbl22)	⁻	†	⁻	nr	(Gille *et al.*, 2011)
	esk1 (tbl29)	⁻	nr	nr	†	(Yuan *et al.*, 2013)
	esk1 (tbl29)	nr	nr	†	†	(Xiong *et al.*, 2013)
AXY9	*axy9*	⁻	†	†	nr	(Schultink *et al.*, 2015)
CE1	*AnAXE*	⁻	⁻	†	nr	(Pogorelko *et al.*, 2013)
	AnAXE1	†	†	†	nr	(Pawar *et al.*, 2015)
CE12	*AnRAE*	†	†	-	nr	(Pogerelko et al., 2013)
CE13	*PtPAE1*	†	nr	†	nr	(Gou *et al.*, 2012)
	VrPAE	†	nr	nr	nr	(Orfila *et al.*, 2012)
	pae8/pae9	†	nr	nr	nr	(de Souza *et al.*, 2014)

"†"= defect in acetylation. "⁻"= no change. "nr" = not reported

According to current model, acetyl CoA is transported via RWA transporter protein from cytoplasm to Golgi but presence of intermediate/another substrate is possible (**Figure 2**). AXY9 protein could act as transferase/esterase upstream of TBL proteins (Schultink *et al.*, 2015), and TBL proteins would finally transfer acetyl groups to specific cell wall polysaccharides. Though the function of different gene families involved in acetylation is deduced form the genetic evidence, biochemical characterisation of these proteins would give more insight about *O*-acetylation mechanism in plants.
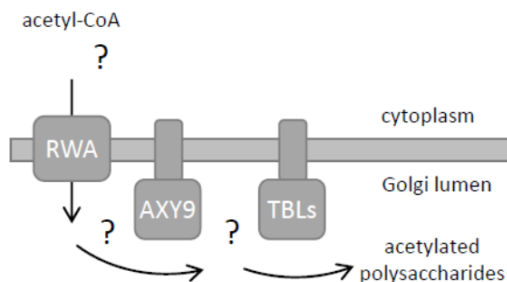
Figure 2. Representative model showing components involved in of *O*-acetylation of polysaccharides (Adapted from (Schultink *et al.*, 2015) (Reproduced with permission))

1.6 Modification of cell wall polysaccharide *O*-acetylation

1.6.1 Acetyl Xylan Esterase (EC 3.1.1.72)

Acetyl xylan esterases (AXE) are involved in hydrolysis of acetyl groups present on xylan chain. AXEs are found in eight different carbohydrate esterase (CE) families: CE1-7 and CE16 (Pawar *et al.*, 2013; Biely, 2012). AXEs are found in wood degrading fungi and bacteria but no AXE activity has been purified from plants. However, there are two putative acetyl xylan esterase genes in Arabidopsis listed in CE6 family (*http://www.cazy.org/CE6_eukaryota.html*). The reaction mechanism, protein structure and substrate specificities of microbial esterases are listed in **Table 4.** AXEs from families CE1, CE5 and CE6 can deacetylate xylan at positions X2, X3 and X23 (Neumuller *et al.*, 2015). CE4 can act on X2 and X3 position. None of AXEs have so far been found to be active on acetyl group at position X3 in a Xyl*p* substituted with meGlcA. These reported activities have been either shown on synthetic substrates or on isolated xylan, so it is possible that these activities might be restricted when these enzymes are heterologously expressed in plants.

1.6.2 Pectin Acetyl Esterase

Pectin acetyl esterases (PAEs) are categorised into CE12 and CE13 families and they are found in bacteria, fungi and plants. PAEs from CE12 are characterised only in bacteria and fungi. These enzymes deacetylate GalA of RG-I or HG (Schols *et al.*, 1990). The crystal structure of rhamnogalacturonan acetylesterase (RGAE) has revealed α/β/α protein folding containing serine–histidine–aspartic acid catalytic triad (Molgaard *et al.*, 2000). PAEs from CE13 family are mainly found in plants. PAEs from Arabidopsis, poplar and mung

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bean have been studied (de Souza *et al.*, 2014; Gou *et al.*, 2012; Orfila *et al.*, 2012). These PAEs can act on acetylated GalA of HG and RG-I, however, RG-I is a preferred substrate for AtPAE8/PAE9 (de Souza *et al.*, 2014). The plant PAEs play important role plant growth, development and defining the cell wall properties.

Table 4. Mechanism of hydrolysis of ester linkages, protein fold and substrate specificity of carbohydrate esterases assigned originally as AXEs. Modified from (Biely, 2012)

CE family	Reaction mechanism/catalytic AAs	Protein fold	Substrate specificities
CE1	Serine esterase	α/β/α sandwich	acetylgalactoglucomannan and acetylated carbohydrates,
	SHD triad		hexosides, cellulose acetate.
CE2	Serine esterase	α/β+β-sheet	hexopyranosyl residue, acetylated xylan and xylosides
	SHD triad		transesterification of hexoses and hexosyl residue
CE3	Serine esterase	α/β/α sandwich	Broad substrate specificity
	SHD triad		
CE4	Metallo-enzymes, aspartic esterases	$(α/β)_7$	specific for acetylated xylan, chitin, peptidoglycan
CE5	Serine esterase	α/β/α sandwich	xylopyranosyl, xyloligosaccharides, glycosides, cellulose acetate
	SHD triad		
CE6	Serine esterase	α/β/α sandwich	acetylated xylan, broad substrate specificity
	SHE triad		
CE7	Serine esterase	α/β	oligosaccharides
	SHE triad		
CE16	Unknown		non-reducing end xylooligosaccharides

1.7 Role of *O*-acetylation in plants

In recent years, substantial progress has been done to understand the role of acetylation in plants by identifying the genes involved in biosynthesis of *O*Ac. There have been also some attempts to modify *O*Ac using microbial acetyl esterases. These mutants have shown either increase or decrease in xylan, XG, mannan and pectin acetylation (Table 3).

Mutation in *TBL27* gene in Arabidopsis leads to complete loss of XG acetylation in vegetative organs but not in acetylation of other polymers (Gille

et al., 2011). This mutant didn't show any defect in growth/development of the plants which signifies that XG acetylation doesn't have direct impact on plant morphology (Gille *et al.*, 2011). Arabidopsis *rwa* mutants show defect in acetylation of many polysaccharides (XG, pectin, mannan, and xylan) (Manabe *et al.*, 2013; Manabe *et al.*, 2011). Growth is not significantly affected in *rwa* single/double mutants but *rwa* triple and quadruple mutants have irregular xylem phenotype, weak stems and overall growth and development defects (Manabe *et al.*, 2013; Lee *et al.*, 2011a). Heterologous expression of *VrPAE* (Table 3) in potato tissues resulted in decreased in pectin acetylation and increase in stiffness and strength of potato cell walls (Orfila *et al.*, 2012). *esk1* Arabidopsis mutants exhibited decrease in xylan and mannan acetylation (Xiong *et al.*, 2013; Yuan *et al.*, 2013). Mutation in *AXY9* gene has strong decrease in xylan acetylation (-90%) and in XG acetylation (-60%). *esk1* and *axy9* both have defect in plant growth and development. Overexpression of poplar *PAE* in tobacco altered plant morphology and led to severe impairment in growth of reproductive organs (Gou *et al.*, 2012). These results suggest that acetylation of xylan, mannan and pectin has major role in plant growth and development. On the other hand, post synthetic modification in acetylation of cell wall polysaccharides using fungal esterase had no impact on plant morphology (Pogorelko *et al.*, 2013). Triple and double Arabidopsis *gux* mutants lacks meGlcA/GlcA on xylan chain but acetyl group substitution has been increased (Chong *et al.*, 2014; Lee *et al.*, 2014). Arabidopsis *rwa* mutant have decreased in xylan acetylation but increase in GlcA/meGlcA attached to xylan (Lee *et al.*, 2014). Surprisingly, dwarf phenotype of *esk1/tbl29* is rescued by expressing GUX1 under the control of TBL29 promoter in *tbl29* plants (*pTBL29::AtGUX1-tbl29*) (Xiong *et al.*, 2015). Reversal of dwarf phenotype suggests that side chains are necessary to have stable xylan chain. This also suggests that substitution in xylan is tightly regulated in Golgi membrane before it is deposited to the cell wall. Modification in acetylation clearly affects the plant morphology but why it triggers such a strong effect on plant is still a question. Digestibility and solubility assays suggest this could be related change in properties of polysaccharides and their interaction with neighbouring polysaccharides. It would interesting to compare structure of xylan in *pTBL29::AtGUX1-tbl29* and wild type with respect to its interaction with cellulose. Molecular dynamics study would be one of the approaches to study such interactions in plants in all above acetylation mutants (Busse-Wicher *et al.*, 2014).

It is known that acetylation of polysaccharides makes hydrolysis of polysaccharides difficult for microbial enzymes (Biely *et al.*, 1985). Surprisingly, *rwa2* mutants having reduced acetylation in different cell wall polysaccharides showed increase in resistance against *Botrytis cinerea.* Deacetylation of xylan by overexpressing microbial AXEs and by mutation in

the genes involved biosynthesis of acetylation was achieved in Arabidopsis and those plants exhibited resistance against biotrophic and necrotrophic pathogens (Pogorelko *et al.*, 2013; Manabe *et al.*, 2011). Both these studies have indicated that effect on susceptibility could be a direct response to an altered acetylation or some other defense responses. Different possible scenarios have been discussed to understand resistance against different pathogens but the exact underlying mechanisms of the increased resistance are not understood. Nevertheless, if trees/crops with modified acetylation have resistance against pathogens, they can serve as potential varieties for bioenergy application.

Removing acetylation chemically and enzymatically increased hydrolysis of polysaccharides (Pawar *et al.*, 2013). But, deacetylation in pectin hindered the digestibility of pectin by pectinases (Gou *et al.*, 2012). The plants with deacetylated xylan can be more accessible to xylanases (Yuan *et al.*, 2013). However, overall increase in sugar yield is not seen in mutants having altered polysaccharides acetylation (Xiong *et al.*, 2015; Pogorelko *et al.*, 2013; Xiong *et al.*, 2013; Lee *et al.*, 2011a). Certainly more studies are necessary to understand effect of acetylation on polysaccharide properties and its role for interacting with neighbouring polysaccharides. This would lead to understanding how to optimise the acetylation for improvement in biomass quality.

1.8   Ways of optimising acetylation in plants

There are different ways to modify the acetylation of cell wall to study its importance in plant growth development and in determining lignocellulosic properties.

As mentioned earlier, different acetyl esterases can hydrolyse acetylation on xylan and pectin but AXE activity have not been shown to present in plants, in such cases heterologous expression of acetyl xylan esterase would be one of the possible ways to modify the acetylation in xylan. As cell wall polysaccharides are acetylated in Golgi, and the acetylated polysaccharides are transferred through vesicle to the cell wall, it is possible to target these AXEs either to Golgi or to plant cell wall using specific signal and retention sequences (Gandla *et al.*, 2015; Pogorelko *et al.*, 2011; Buanafina *et al.*, 2010).

Another mode to modify acetylation is to use known native genes involved biosynthesis of acetylation. These identified genes can be modified in plants by using traditional silencing or overexpression approaches or by a recently developed CRISPR-Cas technology (Shan *et al.*, 2013)*.* In CRISPR-Cas system, a guide RNA fragment specific for target sequences forms a complex with Cas9 endonuclease and cleaves the target DNA. This method can be used to identify the function of target genes or gene families. There are three different gene families (RWA, TBL and AXY9) involved in biosynthesis of

acetylation that could be exploited but they have only been studied in Arabidopsis so far. It is therefore necessary to study these genes in bioenergy crops like aspens, willows, and switchgrass and unravel effects on plant morphology and biomass digestibility.

Acetylation of cell polysaccharides involves multiple components involving many genes responsible for one process. Also, it is only a part of polysaccharide biosynthesis process that could be tightly coupled with other biosynthetic processes involving other genes which are not directly involved in biosynthesis of acetylation. The genomic regions which contain genes associated with complex traits like acetylation are called Quantitative Trait Loci (QTLs) (Collard *et al.*, 2005). Generally, linkage maps are created which depict genetic distances between different DNA markers. These maps are used to identify QTLs for particular trait. The basis of QTL analysis is segregation of genetic markers during meiosis. The tightly linked genes are generally transferred to next generation as a block in contrast to genes which are far from each other on the chromosome. So, to create mapping population two parents are crossed and their progenies are used for phenotyping. Phenotypic variation and genetic variation (Single nucleotide polymorphism, SNP) can be used to determine linked genes/genomic regions contributing to particular phenotype. Different statistical methods like Analysis of Variance (ANOVA), *t*-test and linear regression are used to locate the QTLs. Correlation values help to determine phenotypic variation related to marked gene linked to QTL. Using comparative mapping, QTLs from two species can be compared. For ex. *Salix* and *Populus* belonging to Salicaceae family and having same number of chromosomes and similar genome size, can be used for comparison of QTLs and identification of genes contributing to QTLs (Berlin *et al.*, 2010). Those identified QTLs can be used to incorporate desirable traits for improvement in biomass quality through breeding.

2 Objectives

The main objective of this study was to understand the role of polysaccharide acetylation in plants and to identify ways to optimize the acetylation for biomass improvement, with a special focus on woody biomass. To achieve this, the study was performed on three model species, a herb, *Arabidopsis thaliana*, and two woody species, *Populus tremula* L. *x tremuloides* Michx. (hybrid aspen) and *Salix sp.*(willow). Specific goals for this project are listed below-

➢ Characterisation of xylem specific promoter to modify acetylation specifically in the wood (***Paper I***)
➢ Effect of expression of acetyl xylan esterase (AXE) from *A. niger* in Arabidopsis and in hybrid aspen (***Papers II and III***)
➢ Characterisation of Reduced Wall Acetylation (RWA) family in *Populus* (***Paper IV***)
➢ Identification QTLs region for acetyl content and wood chemical traits in willow (*Salix spp.)* (***Paper V***)

3 Results and Discussion

3.1 Isolation, characterisation and application of hybrid aspen xylem-specific promoter (*Paper I*)

To specifically target acetylation in the wood, it was important to isolate promoter which can express in developing xylem cells. Xylan is one of the abundant polysaccharides present in secondary wall layers of xylem cells. In this project, we characterised the promoters from xylan biosynthetic genes of family GT43, identified the most xylem specific promoter, and compared it with 35S promoter by testing their efficiency in altering wood acetylation.

Glycosyl transferase 43 family (GT43) is involved in xylan backbone synthesis. We analysed 7 members from GT43 (GT43A, -B, -C, -D, -E, -F and -G) to identify expression pattern in different tissues. GT43A and GT43B are highly expressed in xylem tissue (Figure 1c, 1d, *Paper I*) as compared other GT43 members. To confirm this, we cloned 1.5Kb promoters of GT43 genes with GUS reporter and expressed these constructs in hybrid aspen. Histochemical analysis of GUS activity in stem cross-sections in the transgenic lines revealed that GT43A and GT43B genes are expressed in developing xylem fibres, vessel elements, xylem ray cells and phloem fibres (Figure 2a,b, *Paper I*). GT43A and GT43B genes were transactivated by secondary cell wall-inducing transcription factors (*Ptxt*MYB21, PNAC085 and PNAC058) suggesting that these genes are involved specifically in secondary cell wall biosynthesis (Figure 3, *Paper I*).

To test the efficacy of GT43B promoter as compared to *35S* constitutive promoter, overexpression and downregulation constructs were prepared using both promoters, and they were transferred to hybrid aspen. For the overexpression constructs, I used acetyl xylan esterase (AXE) from carbohydrate esterase family 5 (CE5). For the downregulation constructs, all four members of *RWA* genes family were targeted by RNAi methodology using the two promoters as above. CE5 expression was higher in transgenic trees driven by 35S promoter as compared to GT43B promoter (Figure 5a, *Paper I*). On the other hand, no suppression of any of the *RWA* genes was detected in

*35S::RWA-RNA*i lines whereas *RWA-A*, *RWA-B* and *RWA-D* genes were downregulated when using *pGT43B::RWA*-RNAi construct. (Figure 5b, **Paper I**).

Since both overexpression and downregulation constructs should modify wood acetylation in these transgenic lines, the wood of transgenic plants was analysed by FT-IR and by acetic acid release. FT-IR analysis showed that changes in different wavenumbers assigned to acetyl esters were more obvious in *pGT43B::CE5* lines than in *35S::CE5* lines, although expression of CE5 was higher in 35S::CE5 transgenic lines (Figure 6c, 6e, **Paper I**). Similarly, *pGT43B::RWA* RNAi lines showed more prominent changes in ester linkages as compared to *35S::RWA* RNAi lines. (Figure 6d, 6f, **Paper I**). Acetyl content was reduced by 10% and 15% in *35S::CE5* and *pGT43B::CE5* lines, respectively, as compared to WT (Figure 6g, **Paper I**). Acetyl content was unchanged in *35S::RWA* RNAi lines but 17% reduction in acetyl content was observed in *pGT43B::RWA* RNAi lines (Figure 6h, **Paper I**).

In conclusion, expression of *CE5* gene was not higher in *pGT43::CE5* lines as compared to *35S::CE5* but*, pGT43B::CE5* lines showed more effective removal of wood acetylation. On the other hand, *RWA* genes were not downregulated in *35S::RWA RNAi* lines but the effective downregulation in *RWA* genes was seen in *pGT43B::RWA* RNAi lines. This resulted in better modification in wood acetylation in *pGT43::RWA RNAi* lines as compared to *35S::RWA RNAi* lines. Together these results show that, *GT43B* promoter is more useful promoter than *35S* promoter for modification of traits in secondary cell wall.

3.2   Transgenic plants have normal growth with reduction in cell wall acetylation (*Papers II, III & IV)*

All matrix polysaccharides are acetylated. To understand their role in plants growth and development, the cell wall acetylation was modified by expressing fungal AXE in Arabidopsis and hybrid aspen and down regulating *RWA* genes in hybrid aspen.

A. niger AXE1 from CE1 gene family (Table 4) was targeted to the cell wall using native signal peptide (Figure 2a, **Paper II).** *AnAXE1* was expressed using *35S* and hybrid aspen *PtGT43B* promoters in Arabidopsis and using *35S* promoter in hybrid aspen. The cell wall acetylation in Arabidopsis stem was reduced in transgenic lines (*AnAXE1*) as compared to WT (Figures 3b, 3c, and S3b **Paper II**) and the most highly expressing lines showed 32% and 25% reduction in acetylation for *35S* and *PtGT43* promoter constructs, respectively. However, deacetylation in transgenic lines did not affect growth and development (Figures 2d, and S3d, **Paper II**). Transgenic Arabidopsis expressing AXE from *A. nidulans* showed 50% reduction in acetyl content and didn't show any impact on growth (Pogorelko *et al.*, 2013) Similarly, hybrid

aspen transgenic trees expressing *AnAXE1* controlled by *35S* promoter showed a 15% decrease in wood acetylation without any effect on plant growth (Figures 1A, 1D, 1F, S1A, S1B and S1C, **Paper III**). Moreover, downregulation of both *RWA* gene clades in hybrid aspen resulted in 25% decrease in wood acetylation (Figure 4B, 4C **Paper IV**) without any impact on plant morphology, height or diameter of stem (Figure S4, Table S2, **Paper IV**).

Arabidopsis *rwa1/2/3/4, axy9*, and *esk1* mutants showed 65%, 70% and 60% reduction in total cell wall acetyl content, respectively (Schultink *et al.*, 2015; Manabe *et al.*, 2013). This much reduced level of acetylation was mainly due to deacetylation in xylan. These mutants were dwarf and had irregular xylem phenotype. Moreover, % of deacetylation was higher in these mutants as compared to the transgenic Arabidopsis and hybrid aspen expressing AXE (**Papers II, III**) (Pogorelko *et al.*, 2013) and transgenic hybrid aspen *pGT43B::RWA RNAi* lines (**Paper IV**). This suggests that level of acetylation is an important factor determining the impact on plant growth. On the other hand, overexpression of *Populus* PAE in tobacco resulted in 13% to 42% and 8% to 11% decrease in acetylation, in water soluble and acid soluble pectin fractions, respectively, and this resulted in severe defects in growth of organs and overall plant development (Gou *et al.*, 2012). Deacetylation of pectin in potato resulted in increased strength of potato cell walls (Orfila *et al.*, 2012). Whereas the complete deacetylation in XG did not have any effect on growth in Arabidopsis (Gille *et al.*, 2011).

Comparing my results with published data suggests that 50% of deacetylation in cell wall is tolerated by plants, except when deacetylation for in pectins. Indeed, a small alteration in pectin acetylation resulted in strong growth phenotypes, suggesting that pectin acetylation is more important in determining plant growth and development than acetylation in other polymers.

3.3 Effect of reducing cell wall acetylation strategies on acetylation of different polysaccharides in Arabidopsis and hybrid aspen (*Papers II, III & IV*)

AnAXE1 was reported to be active on acetylated xylan but not pectin *in vitro* (Koutaniemi *et al.*, 2013; Kormelink *et al.*, 1993). My aim was to elucidate its effects on acetylation of different polysaccharides when over the enzyme is expressed *in planta*. Moreover, RWA proteins were reported to affect acetylation in many polysaccharides in Arabidopsis in a non-specific fashion, with exception of RWA2, which was more involved in acetylation of pectins and XG (Manabe et al., 2011; 2013; Lee et al., 2011). Therefore, I have studied the role of the two *RWA* gene clades in hybrid aspen in xylan acetylation.

To determine acetylation specifically in the xylan, cell walls of *An*AXE1 expressing Arabidopsis and hybrid aspen and hybrid aspen plants with downregulated *RWA* both clade AB and CD genes were treated with xylanases

and xylooligosaccharides (XOS) were detected by MS. *AnAXE1* transgenic lines have increased in amount of shorter chain XOS (Figures 4a, 4b, ***Paper II***; Figure 2A, S2, ***Paper III;*** Figure 4D**,** Figure S5C**,** ***Paper IV***). This is expected because deacetylation of xylan in transgenic lines or *in vitro* by activity of AXE will create binding sites in xylan facilitating xylanase hydrolysis. These results indicate that xylan of transgenic lines expressing AXE and having suppressed RWA genes are less acetylated and easily accessible to xylanases.

The NMR analysis on DMSO extracted wood samples can also reveal acetylation of xylan and it was used to characterize the xylan in the wood in *An*AXE1 expressing aspen and *RWA* downregulated aspen. Xylan acetylation was reduced in *AnAXE1* expressing aspen transgenic lines. Because of less acetylation at X2 position (Figure 2B, ***Paper III***). It is possible that *An*AXE1 is specific for X2 position. *pGT43B::ABCD*-RWA RNAi transgenic lines showed reduction total acetylation because of decrease in acetylation X2 position (Figure 4E, ***Paper IV***). Surprisingly, acetylation at X3 was increased. This is probably because a decrease in acetylation of xylan makes it more accessible to endogenous xylanases which might change xylan structure in cell wall. This might result in a decrease in pools of deacetylated xylan. This is why RWA RNAi lines show an apparent increase in substitution of acetyl groups on X-3 in Xyl.

 AnAXE1 expressing Arabidopsis plants were also analysed by XG OLIMP and showed deacetylation in XG, suggesting that *An*AXE1 can acts as a XG acetyl esterase *in planta* (Figure 7a, ***Paper II***). AXE from *A. nidulans* when expressed in Arabidopsis did not induce deacetylation in XG despite having 80% identity with AXE1 of *A. niger* at the amino acid level. AXEs from family CE1 are known to have broad specificity and can use even cellulose acetate as substrate so the activity of *An*AXE1 on XG is possible, but it will be necessary to test it using pure enzyme preparation and pure acetylated XG.

Surprisingly, *35S*::*AnAXE1* expressing Arabidopsis plants showed increase in pectin acetylation in the leaves, which was not observed when *AnAXE1* was expressed using the *PtGT43B* promoter (Figure 7b, ***Paper II***). This was accompanied by changes in expression of *RWA* genes in leaves and stem tissues (Figure 8, ***Paper II***). Overexpression of pectin acetyl esterase in tobacco leads to decrease in pectin acetylation but increase in xylan acetylation whereas Arabidopsis plants expressing *A. nidulans* AXE have changes in RWA gene expression (Pogorelko *et al.*, 2013; Gou *et al.*, 2012). This suggests that, plants have some kind of acetylation homeostasis mechanism responding to modification of acetylation in cell wall polysaccharides. It would be interesting to study if same type of acetylation sensing is present in Arabidopsis mutants like *rwa, axy9, esk1*.

To sum up, AXE1 from *A. niger* can hydrolyse acetyl groups on xylan and also probably on XG chain when overexpressed *in planta*. The 2D NMR

analysis exhibited decreased xylan acetylation in *AnAXE1* overexpressing and RWA RNAi transgenic lines because of reduction in acetylation at X-2 position. Deacetylated xylan is more accessible to xylanases which is shown by different pattern of XOS in transgenic lines as compared to WT. Increase in pectin acetylation and change in expression RWA genes was observed in *An*AXE1 overexpressing Arabidopsis. This suggests that plant to respond to modification in cell wall acetylation.

3.4 Effect of reducing cell wall acetylation in matrix polysaccharides on other cell wall components in Arabidopsis and hybrid aspen (*Papers II, III & IV*)

In order to understand the effect of change in acetylation on cell wall properties, different cell wall compositional analyses were performed in transgenic plants expressing *An*AXE1 and transgenic lines having downregulation in RWA genes (**Paper II, III and IV**)

   AnAXE1 expressing Arabidopsis plants did not show any changes in cell wall composition (Supplementary Table 1, 2, **Paper II**).However, transgenic aspen plants expressing *AnAXE1* showed decrease in amount of Xyl, increase in amount of non-cellulosic Glc and increase in crystalline cellulose (Table 2, **Paper III**). Total lignin content was same but decrease in S/G ratio was found transgenic lines as compared to WT (Table 1, Table 2, **Paper III**). Downregulation of both *RWA* clades in hybrid aspen resulted in decrease of Xyl and increase in Glc, GlcA and meGlcA (Table 1, **Paper IV***)*.

   Decrease in Xyl in *AnAXE1* expressing, and in *RWA* suppressed lines is probably related to increase in amount of soluble Xyl or oligosaccharides because of action of plant xylanases (Derba-Maceluch *et al.*, 2015). Also, increase in GlcA and meGlcA in *RWA RNAi* (Table 1, **Paper IV***)* transgenic lines is not surprising because *rwa 1/2/3/4* Arabidopsis mutants show similar increase in GlcA/meGlcA (Lee *et al.*, 2014). This is attributed to a competition for substitution between GlcA/meGlcA and acetyl groups at xylose-2 position. Change in S/G ratio was seen only *AnAXE1* expressing aspen and not *RWA-RNAi* lines (Table 2, **Paper III**; Table S3 **Paper IV**). This suggests that decrease in S/G ratio might not be a direct effect of deacetylation in wood. Investigation needs to be done if it is either because of change in monolignol biosynthetic pathway or change in polymerisation of lignin. Increase in cellulose crystallinity in *An*AXE1 expressing aspen (Table 2, **Paper III**) supports the notion that deacetylation of xylan increases its interaction with cellulose (Busse-Wicher *et al.*, 2014; Kabel *et al.*, 2007a).

   To understand the effect of deacetylation on Lignin-Carbohydrate-Complexes (LCC), different LCC fractions were isolated from *An*AXE1 expressing hybrid aspen lines and compared with similar fractions in WT. The

weight of four sequentially extracted LCC fractions, and in particular, the weight of water-soluble fraction, were increased in transgenic lines as compared to WT (Figure 5A, *Paper III*). Acetyl Bromide Soluble Lignin (ABSL) content was increased in this fraction in transgenic lines as compared to WT (Figure 5B, *Paper III*) indicating that lignin is more soluble in the transgenic lines. Relative carbohydrate and lignin content was analysed in these fractions by Pyrolysis-GC-MS. S/G ratio was lower in all LCC-fractions of transgenic line as compared to WT except in LCC-1 fraction representing lignin associated with pectin (Table S2, *Paper III*). Dioxane water-soluble lignin content was higher in transgenic lines as compared to WT (Figure 4B, *Paper III*). This suggests that solubility of lignin was increased in transgenic lines as compared to WT. Acidified-dioxane soluble lignin fraction in transgenic lines contained more Glc and Xyl than in WT (Figure 5C, *Paper III*). These analyses suggest that, it is easier to extract LCCs from transgenic lines compared to WT because of increased lignin and xylan solubility.

Taken together these results show that xylan deacetylation induced discrete changes in cell wall composition, crystallinity of cellulose and LCCs extraction. It would be further interesting to screen transgenic trees with varying degree of deacetylation and correlating it with different cell wall properties.

3.5 *AnAXE1* expressing Arabidopsis plants showed increased resistance against biotic stress (*Paper II*)

Defect in acetylation of cell wall polysaccharides has led to increase in resistance against different pathogens (Pogorelko *et al.*, 2013; Manabe *et al.*, 2011). Therefore, we addressed the question by testing pathogen response in *An*AXE1 overexpressed transgenic Arabidopsis lines.

Resistance to biotrophic (*Hyaloperonospora arabidopsidis*) and necrotrophic (*Plectosphaerella cucumerina*) pathogens was tested in *AnAXE1* expressing Arabidopsis plants along with *rwa2-3* and *axy4* Arabidopsis mutants. *AnAXE1* expressing plants and *rwa2-3* mutants were found to have increased resistance against *H. arabidopsidis* (Figure 9a, *Paper II*). No change was found in susceptibility against *P. cucumerina*. To test if there was a change in regulation of basal immunity, expression of different immunity-related genes was analysed. The resistant lines showed increased expression of *WRKY40* (W-box containing transcription factor) which was most important in the line expressing *AnAXE1* from *pGT43B* promoter (Figure 9b, *Paper II*). *PR5 (Pathogen Related 5),* induced in response to pathogen attack, was upregulated in all transgenic lines, whereas *PAD3* gene, involved in biosynthesis of phytoalexins, was downregulated in the line that had increased pectin acetylation.

There are different scenarios, which can explain these results. The direct scenario is that deacetylated matrix polysaccharides might be more susceptible to plant endogenous hydrolases, which creates large amount of oligosaccharides (OS). These OS could act as damage-associated molecular patterns (DAMPs), which trigger the defense responses. Production of DAMPs would be different depending on which polysaccharide acetylation is affected. These endogenous elicitors trigger ROS production, biosynthesis of phytoalexins and callose deposition. It is also possible that integrity in epidermal walls might have been affected which led to activation different stress responses. All these acetylation mutants have affected acetylation in different polysaccharides and level of deacetylation is different. That is why it is difficult to uncover possible mechanism for increase in resistance against different pathogens. This problem was partly addressed by comparing *An*AXE1 expressing plants with acetylation mutants *rwa2* and *axy4* suggested that resistance against *H. arabidopsidis* was because of deacetylation of xylan as it was only common change in the mutants.

In conclusion, these results indicate that *AnAXE1* transgenic lines are more resistant to biotrophic pathogen *H. arabidopsidis,* possibly because of activation of basal defense responses triggered by a change in acetylation of xylan.

3.6   Effect of decrease in cell wall acetylation on lignocellulose saccharification properties (*Papers II, III, & IV*)

It has been shown that, synergistic action of AXE and xylanases *in vitro* increases Glc and Xyl yields of lignocellulosic biomass (Zhang et al., 2011b; Selig et al., 2009). This implies that deacetylation of polysaccharides should positively impact on saccharification properties of lignocellulose. However, none of acetylation mutants, showed any positive effect on saccharification (Xiong *et al.*, 2015; Xiong *et al.*, 2013; Lee *et al.*, 2011b; Pogorelko *et al.*, 2011). To understand the effect of reduced acetylation *in planta* on saccharification, we performed digestibility and solubility tests by different methods.

Improvement in sugar yields of saccharification after different pretreatments was found in Arabidopsis transgenic lines expressing *An*AXE1 using both *35S* and *PtGT43B* promoters pretreatment (Figures 5, and S4, *Paper II*). Similar to Arabidopsis, *AnAXE1* expressing hybrid aspen showed improvement in Glc yield without pretreatment (+37%) and after acid pretreatment (+14%) (Figure 3, *Paper III*). Predominant increase in Glc yield was because of increase in accessibility for cellulose, as shown by increase in Glc after enzymatic hydrolysis (Figure 3B (Insert), *Paper III*). Yield of all sugars in saccharification without pretreatment was increased by 20% *pGT43B::ABCD-RWA* RNAi transgenic trees in comparison with WT (Figure

5A,, ***Paper IV***). Increase in Glc and Xyl conversion rates indicates enhanced accessibility for cellulose and xylan in *pGT43B::ABCD-RWA RNAi* transgenic lines *(*Figure 5C, ***Paper IV).* Slight increase in Glc was seen after acid pretreatment because of increase in cellulose accessibility (Figure 5B, 5D, ***Paper IV***). These results suggest that modification in acetylation of polysaccharides improves biomass digestibility without or after different pretreatments

Interestingly, transgenic Arabidopsis lines expressing *AnAXE1* with relatively small decrease in acetylation (by 10% or less) showed similar improvement in sugar yields with alkaline pretreatment as the lines with 25%-30% reduction of acetyl content (Figure 5, ***Paper II***). Similarly, *pGT43B::ABCD-RWA* RNAi lines (***Paper IV***) showed more reduction wood acetyl content but less improvement in sugar yield both with and without pretreatment as compared to *AnAXE1* expressing aspen (***Paper III***). This suggests that too strong deacetylation of polysaccharides might not be desirable for saccharification yields. For example, deacetylation of xylan may result in its precipitation or increase in hydrogen bonding with neighbouring polysaccharides including cellulose, which will negatively affect digestibility. This could be one of the reasons why Arabidopsis acetylation mutants having severely decreased acetyl content did not show improvement in sugar yield (Xiong *et al.*, 2013; Lee *et al.*, 2011a; Pogorelko *et al.*, 2011).

Increase in amount of Xyl, and Glc in water, acidic and alkaline pretreatment liquids was found in transgenic Arabidopsis expressing *AnAXE1* suggesting solubility of xylan and some other polysaccharides was increased in them compared to WT (Table 1, ***Paper II***). Aspen expressing *AnAXE1* showed also increase in Glc but in contrast to Arabidopsis they showed a decrease in Xyl in acid pretreatment liquid (Figure 3 C, ***Paper III***). This is not surprising since they exhibit a decrease in Xyl content and an increase in non-crystalline Glc content (Table 2, ***Paper III***). Decrease in Ara, Xyl and Man in acid pretreatment liquid was seen in *pGT43B::RWA*-RNAi transgenic aspen. Monosaccharide composition confirms less Xyl in *pGT43B RNAi* lines (Figure 5B, ***Paper IV).*** Since no change in content of Ara and Man was observed, it is possible that solubility of these sugars might have been reduced because of modification in acetylation (Figure 5B, ***Paper IV).*** Decrease in xylan acetylation in Arabidopsis increases solubility of Xyl or Glc (Table 1, ***Paper II***) but not hybrid aspen.

Xylan extracted from *AnAXE1* expressing plants and hybrid aspen RWA downregulated lines is more easily digested by endoxylanase *in vitro* as compared to WT (Figure 4AB, ***Paper II;*** Figures 2A and S2*,* ***Paper III;*** Figures 4D and S5C*,* ***Paper IV***). This suggests that xylan in these transgenic plants might be also more susceptible to plant endogenous GH10 endoxylanases/endotransglycosylases (Derba-Maceluch *et al.*, 2015) and xylan

attached to lignin is predicted to be easier to extract as explained in **Figure 3**. This is supported by our results where transgenic hybrid aspen trees exhibited increase in solubility of lignin in dioxane-water (Figure 4B, *Paper III*) and in water (Figure 5B – LCC-X fraction, *Paper III*).

In conclusion, the data from three different studies (*Papers II, III, and IV*) indicate that reduction of cell wall acetylation in plants is a promising approach to reduce recalcitrance of biomass. They also point out that level of deacetylation is important in determining saccharification properties.
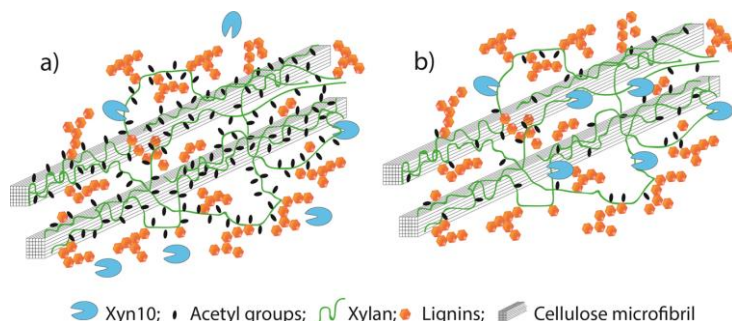
Figure 3. Hypothetical model showing the action of endogenous GH10 endoxylanase/xylan endotransglycosidase Xyn10A in **a)** wild type aspen, and **b)** transgenic aspen with reduced xylan *O*-acetylation. Figure provided by Marta Derba-Maceluch.

3.7 Understanding the functional diversification of RWA genes in *Populus* (*Paper IV*)

Genes involved in biosynthesis of acetylation have been identified in *Arabidopsis thaliana*. Function of those genes has not been investigated in woody species. The aim of this project is to elucidate the role RWA genes in wood acetylation in *Populus*.

There are four RWA genes in both, *Populus and* Arabidopsis species, named *PtRWA-A - PtRWA-D*, and *AtRWA1 - AtRWA4*, respectively. Phylogenetic analysis revealed that *PtRWA-A, and PtRWA-B* are closely related to *AtRWA-1, -3 and -4,* and *PtRWA-C* and *PtRWA-D* are closely related to *AtRWA-2*. It has shown that RWA2 Arabidopsis protein has similar biochemical function to RWA1, -3, and 4 forming a separate clade, as phenotype of quadruple *rwa* mutants reverted back to normal when RWA2 was overexpressed in the quadruple mutant background (Manabe et al., 2013).

Transmembrane topology modelling suggests that *Populus* RWA proteins have 10-12 transmembrane domains, which is similar to Arabidopsis RWA proteins (Lee *et al.*, 2011b) (Figure S1B, *Paper IV*). *Populus* RWA proteins

have conserved Cas1p domain and high amino acid similarity to Arabidopsis RWA proteins (Figure S1, *Paper IV*). As Arabidopsis *rwa* mutants showed defect in acetylation of pectins, XG, xylan and mannan, the encoded proteins were hypothesized to be acting as transporters for acetyl-CoA serving for acetylations in these different polysaccharides (Gille, 2012).

Promoter transactivation analysis of four *RWA* genes of *Populus* was done using secondary cell wall specific transcription factors *Pt*NST1 and *Pt*MYB21. *RWA-A* and *RWA-B* promoters were highly transactivated by these transcription factors. Small but significant transactivation was observed in *RWA-C* promoter but *RWA-D* promoter was not activated (Figure 1B, 1C, *Paper IV*). This indicates that *RWA-A*, *RWA-B* and *RWA-C* could be involved in acetylation of secondary cell wall polysaccharides.

To test if the function of the two *Populus* clades differs, we downregulated clade AB and clade CD individually using *35S* promoter (Figures 2A, 2B, *Paper IV*). Acetyl content was reduced in the wood of both genotypes (Figure 3A, *Paper IV*). Change in a pattern of acidic and neutral XOS was observed in both genotypes when cell wall was digested with xylanase (Figure 3B, 3C, Figure S5A, S5B, *Paper IV*). This suggests that transgenic lines carrying both RWA RNAi constructs have reduction in wood and xylan acetylation.

In conclusion, *Populus RWA-A* and *RWA-B* genes and to some extent *RWA-C* are involved in secondary cell wall biosynthesis. Both AB and CD *RWA* gene clades are responsible for wood and xylan acetylation.

3.8 Identification of QTLs for different wood chemical traits and acetyl content in willow (*Paper V*)

Willow (*Salix spp.*) can be used as a potential feedstock for biofuel production because it has 3-5 years of rotation time, providing 20–tonnes/ha/yr (Szczukowski *et al.*, 2002) and the yield can further be increased through breeding. To utilize the woody biomass in an optimal way, it is necessary to identify heritable wood traits, which can be targeted for breeding to improve the biomass quality. In this project, we used the mapping population of 463 F1 progenies of a hybrid *Salix viminalis* L. × *Salix schwerinii* E. Wolf and *S. viminalis* planted at two locations to determine wood chemical traits using FT-IR, and acetyl content analysis.

Heritabilities of FT-IR signals were the highest for signals assigned to lignin and cellulose/hemicellulose, reaching 43% and 30%, respectively (Tables 1 and 2, *Paper V*). 10%, 21% and 28% of heritability was found for signals assigned for acetyl ester linkages at 1740 cm^{-1}, 1240 cm^{-1} and 1370 cm^{-1} , respectively (Tables 1 and 2, *Paper V*). 0% heritability was found for acetic acid, probably because of batch to batch experimental variation. It is possible that heritability for acetic acid could be observed if single batches were used to calculate heritability.

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Some of the measured chemical traits were found correlated. Strong positive correlation was found between acetyl content, S or G lignin, cellulose/hemicellulose and lignin signals (Table 3, ***Paper V***). Altogether, 28 QTLs for the different FT-IR wavenumbers were identified on 12 Salix chromosomes, explaining between 3.4 % to 6.9 % of variation (Figure 2, ***Paper V***). Three QTLs were identified for wavenumber 1170 cm^{-1} (acetyl) but none was found for wavenumber 1743 cm^{-1} (acetyl) (Table 4, Figure 2, ***Paper V***). Several QTLs were observed for S-lignin, G-lignin, and cellulose/hemicellulose. Co-location of different QTLs for lignin and hemicellulose/cellulose and water was revealed at LGXIV. Also, colocation of QTLs for acetyl signal and S-lignin signals was also observed at LGIX (Table 4, Figure 2, ***Paper V***). DNA sequences from identified QTLs regions were BLAST searched against *Populus trichocarpa* genome database to find corresponding *Populus* gene models which were analysed for similarity with known cell wall related candidate genes (Supplementary Table 1, ***Paper V***). Based on annotations of these *Populus* genes, no genes were identified with function in biosynthesis of acetylation. However, majority of genes did not have any annotation or were similar to expressed proteins of unknown function. Interestingly, several gene clusters related to pectin metabolism and methyl esterification, cellulose biosynthesis, and lignin biosynthesis were identified in vicinity of different QTLs.

In conclusion, we found 3-43% heritability in hybrid *Salix* population for different cell wall chemical traits. In addition, we identified 28 QTLs related to different FT-IR wavenumbers, which correspond to acetyl, S-lignin, G-lignin, S/G ratio, and cellulose/hemicellulose in hybrid *Salix* population. These QTLs contribute to 3.4-6.9% wood phenotypic variation suggesting that this is a promising step towards identification wood chemical traits in willow, which can be further exploited to develop improved willow varieties for biofuel production.

4 Conclusions and Future perspectives

In Paper I, a xylem-specific promoter *pGT43B* has been identified, for modifying traits related to secondary cell wall. This promoter was shown to successfully modify acetylation in Arabidopsis (Pawar *et al.*, 2015) and in *Populus* (Ratke *et al.*, 2015) using both, overexpression and downregulation constructs.

pGT43B promoter can be useful for modification of cell wall polysaccharides residing in the secondary cell wall. Cellulose, lignin and xylan are important components of secondary cell wall of woody plants. These polymers contribute positively or negatively to biomass processibility for biofuel production (as explained in the introduction). It would be interesting to alter genes, involved in biosynthesis of these polymers using *pGT43B* promoter.

In paper II and III, I have shown that it is possible to modify acetylation in cell wall by expressing a fungal gene, *AnAXE1*, without affecting plant growth and development. Transgenic plants expressing *AnAXE1* showed decrease in xylan and XG acetylation. This is the first report showing deacetylation of XG when AXE is expressed in plants. It prompts testing of *An*AXE1 activity *in vitro* on acetylated XG substrate.

Recent finding of CE6 members in Arabidopsis indicates that AXE activity might also be found in plants. It is necessary to identify the type of enzymatic activity of CE6 esterases and their role in plant cell wall modification and biosynthesis. Native AXE encoding genes could be further explored for biomass improvement. It is worth noting that plant pectin acetyl esterase is present and plays important role plant growth and development (Gou *et al.*, 2012; Orfila *et al.*, 2012).

Increase in saccharification in transgenic trees overexpressing *AnAXE1* shows that this is a promising approach to engineer the plants for biofuel production. These results were obtained when trees were grown in the greenhouse and now it is necessary to study effects when they are grown in the field. Importantly, different Arabidopsis cell wall acetylation mutants showed

resistance against different pathogens (Manabe *et al.*, 2013; Pogorelko *et al.*, 2013) (***Paper II***). It is necessary to test if resistance against different pathogens is altered, when acetylation modified hybrid aspen plants are grown in open environment in the field conditions.

Also, it is also important to understand why the saccharification is improved when cell wall acetylation is altered. One way to address this problem is to analyse lignin-associated carbohydrates, and linkages involved in these interactions. Preliminary data from my studies suggest that lignin from transgenic trees is easier to dissolve. The proposed model implicated Xyn10A activity in cleaving deacetylated xylan with lignin attached to it (***Figure 3***). The proposed model can be tested by analysing molecular weight of xylan in cell wall of *An*AXE1 expressing plants.

To understand how acetylation affects interactions in cell wall, it is necessary to study these interactions *in vitro* by the binding assay (Kabel *et al.*, 2007b; Zykwinska *et al.*, 2005). The use of molecular modelling to understand cell wall structure was recently demonstrated to understand the impact of xylan acetylation on its structure and interaction with cellulose (Busse-Wicher *et al.*, 2014). Combining *in vitro* interaction assays, molecular modelling and analysing different acetylation mutants, would not only give the idea about different interactions in cell wall but also help to optimise lignocellulose for different biotechnological applications.

In *Paper IV,* I identified four RWA genes in *Populus,* and demonstrated that three of them RWA-A, -B and -C are active during secondary wall biosynthesis. Both *Populus* RWA gene clades were shown to be responsible for wood and xylan acetylation. Since, RWA-D was not transactivated by secondary cell wall specific transcription factors, it is necessary to study role *Populus RWA-D* in biosynthesis of acetylation. This is the first report in woody plants on biosynthesis of *O*-acetylation.

Future work is needed to understand the role of RWA genes for pectin, XG and mannan acetylation. Complete deacetylation of was not possible in *rwa1/2/3/4* Arabidopsis mutants (Manabe *et al.*, 2013) suggesting possibility of another transporter gene family. TBL and AXY9 are two proposed families involved in acetylation of Arabidopsis. It is necessary to characterise function of these genes in *Populus*. Since TBL is a large gene family, recent techniques like CRISPR-Cas system can be used to target multiple genes to understand their role in *Populus*. Apart from this, biochemical characterisation of RWA, TBL and AXY9 proteins, tissue- and cell-specific expression analysis, and intracellular localization are necessary to determine their exact role during biosynthesis of acetylation.

In *Paper V,* several QTLs in willow were identified for FT-IR signals assigned to acetyl esters, S-lignin, G-lignin, S/G ratio, cellulose/ hemicellulose and water.

Pyrolysis GC-MS is an effective method to determine the chemical fingerprint of cell wall and composition of lignin and it could be used to verify some of the QTLs identified by FT-IR.

Acetyl content was not a heritable trait according to our study. Since, acetic acid measurement was done in batches, it would be interesting to calculate heritability in individual batches and its correlation with acetylation peaks from FT-IR.

Next step would be to determine the saccharification potential and biogas production in these *Salix* clones and correlate these traits with FT-IR chemotypic traits identified in this project. Strongly correlated traits can be useful for breeding with a goal to develop varieties with efficient bioenergy conversion.

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