The role and synthesis of β1,3-galactans in plant cell wall formation

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Abstract

The plant cell wall associated hydroxyproline rich glycoproteins (HRGPs) play an important role in cell wall formation, but the mechanisms are not well understood. The HRGP family consists of the heavily glycosylated arabinogalactan proteins (AGPs) and the moderately glycosylated extensins (EXTs) and proline rich proteins (PRPs). The HRGP glycans are functionally import and investigation of their synthesis provides a way to elucidate HRGP function. In my PhD thesis I studied enzymes with putative functions in AGP glycosylation and cell wall formation.

I show that the two GLYCOSIDE HYDROLASE 43 (GH43) family enzymes of *Arabidopsis thaliana* are Golgi localized β-1,3-galactosidases involved in cell wall biosynthesis. The *gh43null* mutant displays root cell expansion defects when grown on media supplemented with glucose. This phenotype is accompanied by increased cell wall matrix association of AGPs based on β-Yariv quantification and comprehensive microarray polymer profiling of sequentially extracted cell walls. My findings suggest that the loss of exo-β-1,3-galactosidase activity changes the cell wall extensibility in roots, which may be explained by the involvement of the GH43s in AGP glycan biosynthesis.

I also investigated two members of the Arabidopsis glycosyl transferase family 31 (GT31), named *GALT7* and *GALT8* here. *galt7galt8* exhibits severe growth defects and 30% cellulose reduction in both primary and secondary cell walls. Cellulose biosynthesis rate in *galt7galt8* is reduced, but this is not due to reduced CESA transcript or protein levels. Both GALT7 and GALT8 proteins are Golgi localized and a recombinant GALT8 shows galactosyltransferase activity. Quantification of membrane and cell wall associated proteins using mass spectrometry revealed that *galt7galt8* has reduced levels of a subgroup of fasciclin-like AGPs (FLAs). I hypothesize that GALT7 and GALT8 are involved in glycosylating this subgroup of FLAs and that a defect in their glycosylation reduces the rate of cellulose biosynthesis.

The role of HRGPs in wood and wood formation in trees is poorly understood. A total of 157 *HRGP*s are expressed during secondary growth and wood formation in *Populus tremula*. To facilitate functional studies of these *HRGPs* I analyzed their phylogeny and expression during wood formation as well as enzymes putatively involved in HRGP glycosylation. Many of the genes show tight spatio-temporal expression patterns, indicative of specific functions during wood formation. Immunolabeling of mature wood with AGP and EXT glycan specific antibodies unveiled cell type specificity of different epitopes. These data were accompanied by β-Yariv characterization of AGPs and immunoblotting of AGP/EXT glycan epitopes in phloem/cambium, developing wood and mature wood fractions. This revealed differential glycosylation between different wood tissues. Combined the data document the diverse changes in HRGP related processes during wood formation at both gene expression and HRGP glycan biosynthesis level, and associate specific HRGPs and GTs to specific developmental processes during wood formation.

Keywords: Glycoside hydrolase 43 (GH43), Glycosyl transferase 31 (GT31), Arabinogalactan proteins (AGPs), hydroxyproline rich glycoproteins (HRGPs), Cellulose biosynthesis, Cell expansion, Glycosylation, Golgi apparatus

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To my family

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 **Pieter Nibbering** , Bent L Petersen, Mohammed Saddik Motawia, Bodil Jorgensen, Peter Ulvskov, Totte Niittyla* (2020) Golgi-localized exo-beta1,3-galactosidases involved in cell expansion and root growth in Arabidopsis. J Biol Chem 295: 10581-10592

II **Pieter Nibbering**, Gunnar Wingsle and Totte Niittylä* (2020). Loss of Golgi-localized GALT7 and GALT8 reduces the rate of cellulose biosynthesis in Arabidopsis (Manuscript)

III Tayebeh Abedi ¶, Romain Castilleux ¶, **Pieter Nibbering** ¶ and Totte Niittylä* (2020). The distribution and function of cell wall-associated glycoproteins during wood formation in *Populus* (Submitted)

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The contribution of Petrus Nibbering to the papers included in this thesis was as follows:

I Planning and performing experiments, analysing data, contributions to writing and formatting the manuscript

II Planning and performing experiments, analysing data, contributions to writing and formatting the manuscript

III Planning and performing experiments, analysing data, contributions to writing and formatting the manuscript

Abbreviations

ABA	Abscisic acid
AFM	Atomic force microscope
AG-I	Arabinogalactan type I
AG-II	Arabinogalactan type II
AGM	Arabinogalactan methylesterase
AGP	Arabinogalactan proteins
APAP1	ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1
ATPase	Adenylpyrophosphatase
BC1	Brittle Culm1
BFA	Brefeldin A
BLAST	Basic Local Alignment Search Tool
CESA	Cellulose synthase
CMF	Cellulose microfibril
COB	COBRA
COBL	COBRA-LIKE
CoMPP	Comprehensive Microarray Polymer Profiling
CRD	Carbohydrate recognition domain
CSC	Cellulose Synthase Complex
CSI	CELLULOSE SYNTHASE-INTERACTING
DUF	Domain of unknown function
EM	Electron microscope
ER	Endoplasmic Reticulum
EXAD	EXTENSIN ARABINOSE DEFICIENT TRANSFERASE
EXT	Extensins
FLA	Fasciclin-like arabinogalactan protein
FUT	FUCOSYLTRANSFERASE

GALT	GALACTOSYLTRANSFERASE
GFP	Green Fluorescent Protein
GH	Glycoside hydrolase
G-Layer	Gelatinous layer
Glcat14	β-glucuronyltransferase family 14
GPI	Glycosylphosphatidylinositol
GT	Glycosyltransferase
GXM	Glucuronoxylan methyltransferase
HF	Hydrogen fluoride
HG	Homogalacturonan
HPAT	Hydroxyproline-O-arabinosyltransferase
HPGT	hydroxyproline-O-galactosyltransferase
HRGPs	Hydroxyproline rich glycoproteins
Hyp	Hydroxylated proline
kDa	Kilodalton
KOR	KORRIGAN
Le^a	Lewis a
LRX	LEUCINE-RICH REPEAT/EXTENSIN
MALDI-TOF	Matrix-assisted laser desorption/ionization- time of flight
MAP	Microtubule-associated proteins
MUR	MURUS
NMR	Nuclear Magnetic Resonance
NST	Nucleotide sugar transporters
P4H	Prolyl-4-hydroxylases
PERK	PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE
POM	POM-POM
POPGENIE	*Populus* Genome Integrative Explorer
PR1	PATHOGENESIS-RELATED 1
PRP	Proline Rich Protein
Ptt	*Populus tremula x Populus tremuloides*
RAT1	Resistant to Agrobacterium transformation
RAY1	REDUCED ARABINOSE YARIV 1
RFP	Red Fluorescent Protein
RG-I	Rhamnogalacturonan Type I
RG-II	Rhamnogalacturonan Type II
RRA	REDUCED RESIDUAL ARABINOSE
SGT	Serine-O-galactosyltransferase

SOS5	Salt oversensitive 5
$SP_{(3,4,5)}$	Serine and Hyp repetition motif
T-DNA	Transfer-deoxyribonucleic acid
TEM	Transmission electron microscope
TGN	Trans Golgi Network
TLC	Thin Layer Chromatography
TM	Transmembrane
UDP	Uridine diphosphate glucose
UGE	UDP-D-glucose 4-epimerase
UXS	UDP-glucuronic acid decarboxylase
UXT	UDP-XYLOSE TRANSPORTER
WAK	Wall associated kinase
WT	Wildtype
XEH	Xyloglucan endohydrolases
XET	Xyloglucan endo-transglycosylases
XT1	Xylosyltransferase 1
XTH	Xyloglucan endo-transglycosylase/hydrolase
XXT	Xyloglucan xylosyltransferase
Y2H	Yeast two hybrid
YFP	Yellow Fluorescent Protein

1 Introduction

Plants are essential for life on earth. Plants (and other photosynthetic organisms) are able to capture energy from sun light by performing photosynthesis, which converts carbon dioxide and water into carbohydrates and oxygen. This provides the energy and oxygen necessary to maintain life on earth. Plants are crucial for fighting the current climate change, by fixing carbon, preventing desertification, cooling down land surface and preserving biodiversity. Optimizing plant growth for food production, building materials and biomass production is of great importance for economic prosperity and a key tool in the mitigation of climate change. For these reasons, understanding the underlying molecular mechanisms that make it possible to optimize plant growth is critical.

1.1 The plant cell wall

Terrestrial plants fix around 50 billion tonnes of carbon per year (Field et al., 1998). The majority of the fixed carbon in plant biomass is stored in plant cell walls. Cellulose is the main component in most plant cell walls, accounting on average for 40-50% of the plant cell wall dry weight (Rowell et al., 2005). This makes cellulose the most abundant organic polymer on earth. Besides cellulose, hemicelluloses (20-30%) and lignin (20%) are the two other main polymers of the plant cell wall (Robinson, 1990, Schadel et al., 2010). Many plant cell walls also contain glycoproteins and pectin, which can on average account for 5-10% and 5% of the dry weight, respectively (Voragen et al., 2009, Nguema-Ona et al., 2014). Angiosperms are the largest and most diverse group of the plant kingdom. Angiosperms are flowering plants that bear seeds in their fruits and are divided into monocots and dicots, which means that the angiosperm embryo has either one (mono) or two (di) cotyledons. In this thesis I will mainly focus on the cell wall structures of dicots, and specifically the eudicots.

1.1.1 Primary cell wall

The primary cell wall forms an extensible layer surrounding all plant cells emerging from meristems. It contains (dry weight) polysaccharides (90%), structural glycoproteins (2-10%) , phenolic esters (2%), ionically and covalently bound minerals (1-5%) and enzymes (O'Neill and York, 2018). The primary cell wall can contain up to 70% of its total fresh weight in water. The polysaccharides can be subdivided into cellulose (20-30%), hemicelluloses (20-30%) and pectin (20-30%). Cellulose is the main load bearing polymer of the primary cell wall (Anderson et al., 2010) and in the primary cell wall, cellulose microfibrils (CMF) are surrounded by hemicelluloses and pectin.

The primary wall hemicelluloses can be divided into xyloglucan (20-25%), (glucurono) arabinoxylan (5%) and glucomannan (3-5%) (Scheller and Ulvskov, 2010). Xyloglucan consists of a β-1,4-glucan backbone with α-1,6-xylose side chains (Zabotina, 2012). These xylose side chains can be further substituted with different combinations of galactose, arabinose, xylose or fucose. Fry et al. (1993) introduced a single letter nomenclature to describe the different patterns of the side chains of xyloglucan. Most of the vascular seed-bearing plants synthesize XXXG-type xyloglucan (Hoffman et al., 2005). This means that most of the xyloglucan has repeats of 3 glucose units substituted with a xylose (X) and one unsubstituted glucose unit (G). The XXXG pattern can be further substituted with different sugars, which can vary between different tissues and developmental stages (Pauly et al., 2001).

The other main primary cell wall matrix polysaccharide pectin consist of homogalacturonan (HG), xylogalacturonan, apiogalacturonan, rhamnogalacturonan type I (RG-I), and rhamnogalacturonan type II (RG-II) (Harholt et al., 2010). HG is the most abundant pectin and consists of approximately 65% of the total pectin (Mohnen, 2008). HG is a linear α-1,4-linked galacturonic acid, which is partially methylesterified at the C6 and can be *O*-acetylated at O-2 or O-3. RG-I is the second most abundant pectin, which accounts for 20-25% of pectin and consists of a α-1,4-D-galacturonic acid-α-1,2-L-rhamnose backbone with a variety of side chains (Mohnen, 2008). The side chains include galactans and arabinans as well as arabinogalactan type I (AG-I), and arabinogalactan type II (AG-II) polysaccharide chains (Yapo, 2011). AG-II side chains might come from arabinogalactan proteins (AGPs), which will be discussed in section 1.5. RG-I is considered to be one of the most complex parts of the (primary) cell wall, because of the broad range of possible side chains on the RG-I backbone. The other rhamnogalacturonan, RG-II, makes up around 10% of pectin and consists of at least 8 consecutive 1,4-linked α-D-galacturonic acid residues decorated with side branches consisting of 12 different sugars in over 20 different linkages (Harholt et al., 2010).

1.1.2 Secondary cell wall

Secondary cell wall is formed after cell expansion in specialized cell types onto the inner side of the primary cell wall (Kumar et al., 2016). The secondary cell wall physically supports upright plant growth and provides necessary mechanical strength in water transporting cells. The building blocks of the secondary cell wall are cellulose (40-80%), hemicelluloses (10-40%), lignin (5-25%) and small amount of cell wall proteins (Kumar et al., 2016). In dicotyledonous plants glucuronoxylan is the predominant hemicellulose in the secondary cell wall, while mannan and galactoglucomannen are minor components (Scheller and Ulvskov, 2010). Glucuronoxylan consists of a β-1,4-xylose backbone, with α-1,2-linked glucuronosyl and 4-*O*-methyl glucuronosyl substitutions. Lignin is a complex amorphous natural biopolymer, consisting of oxidatively coupled monolignols (Feofilova and Mysyakina, 2016). These monolignols consist p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). Dicots predominantly contain S/G lignin and trace amounts of H lignin (Pattathil et al., 2015).

1.1.3 Plant cell wall glycoproteins

Plant cell wall associated glycoproteins consist of *N*-glycosylated and *O*-glycosylated proteins. Glycoproteins can be found both in the primary and secondary cell wall. *N*-glycosylated proteins are first glycosylated on the asparagine amino acid on the protein backbone with an *N*-Acetylglucosamine, onto which more complex glycosylation is added (Strasser, 2016). *N*-glycosylation plays a role in protein folding, stability, protein-protein interactions and glycan dependent quality control processes in the endoplasmic reticulum (ER) (Strasser, 2016). The cell wall associated *O*-glycosylated hydroxyproline-rich glycoproteins (HRGPs) can be glycosylated on serines and hydroxylated prolines (Hyp) on their protein backbone. The HRGPs are divided in abundantly glycosylated AGPs, moderately glycosylated extensins (EXTs) and less glycosylated proline rich proteins (PRPs) (Showalter et al., 2010).

EXTs are generally described as plant cell wall glycoproteins that act as scaffolds for the deposition of the primary cell wall (Lamport et al., 2011, Moller et al., 2017). The Arabidopsis genome contains 59 EXTs, which are divided into classical EXT, short EXT, chimeric EXT and AGP/EXT hybrids (Showalter et al., 2010). The protein backbone of EXT typically contain two or more serine and four Hyp repetition motifs (SP_4), which can vary in length from SP_3 to SP_5 (Kieliszewski and Lamport, 1994, Showalter et al., 2010). These repetitive motifs are glycosylated by a range of glycosyltransferases residing in the Golgi apparatus. The first serine is glycosylated with one α-4-galactose by serine O-galactosyltransferase (SGT) (Saito et al., 2014). The hydroxyprolines are further glycosylated with a β-4-arabinose by Hydroxyproline-O-arabinosyltransferases

(HPATs) (Ogawa-Ohnishi et al., 2013). This arabinose is then glycosylated with a β-1,2-arabinose by REDUCED RESIDUAL ARABINOSE (RRA), which in turn is glycosylated with another β-1,2-arabinose by XYLOGLUCANASE 113 (Egelund et al., 2007, Gille et al., 2009). The fourth α-3-arabinose is added by EXTENSIN ARABINOSE DEFICIENT TRANSFERASE (EXAD) (Moller et al., 2017). The enzyme(s) that transfer the fifth arabinose are currently unknown. Analysis of EXT glycosylation mutants has established that extensin glycans play roles in cell expansion, root hair growth, pollen tube growth, senescence, flowering initiation, cell wall thickening and plant defence (Ogawa-Ohnishi et al., 2013, Gille et al., 2009, Velasquez et al., 2011, Castilleux et al., 2020).

Some EXTs may be covalently bound to the cell wall matrix. When Qi et al. (1995) sequentially treated cell walls of suspension cultures of *Gossypium hirsutum* with endo-polygalacturonase, cellulase, hydrogen fluoride (HF) hydrolysis and ammonium carbonate, only sugars indicative of RG-I and some protein remained insoluble. It was hypothesized that a covalent linkage between protein(s) and RG-I could not have been an arabinofuranan linkage, since this should have been broken by HF at -73°C (Qi et al., 1995). After a trypsin digest of this residue, 80% of the Hyp was removed and 3 abundant peptides were identified. These peptides were annotated as EXT fragments. However, I found that when using the current Basic Local Alignment Search Tool (BLAST) tool the SPPPPPPPSPPL peptide identified corresponds to a fasciclin-like arabinogalactan protein 21 (FLA21) protein from *Gossypium hirsutum*. Therefore, it is possible that at least one of the proteins identified was not an EXT, but GhFLA21. In support of this, AGP glycosylation does not break with HF at -73°C. Another more recent study performed trypsin digest on pectin extracted from sugar beet pulp and found that some of the identified peptide sequences corresponded to EXT proteins (Nunez et al., 2009). These results suggest that some FLAs and EXTs can be covalently bound to pectin in the cell wall or are at least tightly associated with the cell wall.

The Arabidopsis genome contains 18 genes classified as PRPs (Showalter et al., 2010). Of these, 12 are annotated as classical PRPs and 6 are classified as chimeric PRPs. The PRPs are only modestly glycosylated, and their glycan structures are unknown (Datta et al., 1989, Showalter, 1993). PRPs are associated with plant stress responses and are differentially expressed under many biotic and abiotic stresses (Showalter et al., 2010).

1.2 Assembly of cell wall carbohydrates

1.2.1 Biosynthesis of cellulose

Cellulose is synthesized at the plasma membrane by cellulose synthase proteins (CESA), which form a cellulose synthase complex (CSC). The CSC is assembled in the Golgi apparatus and is then transported via the secretory pathway to the plasma membrane (Haigler and Brown, 1986, Crowell et al., 2009). In the primary cell wall CMFs are deposited transversely to the cell axis, and reorient upon cell expansion to a longitudinal direction (Anderson et al., 2010). Upon cell expansion, new cellulose is deposited in the cell wall, which leads to multiple angled CMF layers in the primary cell wall.

The catalytic core of the CSC consists of CESA1/3/6 alternatively CESA6-like during primary cell wall cellulose biosynthesis and of CESA4/7/8 during the secondary cell wall cellulose biosynthesis (Persson et al., 2007, Taylor et al., 2003). It was shown that CESA4/7/8 exist in 1:1:1 stoichiometry in Arabidopsis stems and based on this stoichiometry and spectroscopic analyses of the CMF thickness indicating that there are 18 glucan chains per elementary CMF, it was proposed that the CSC complex contains 18 CESAs (Hill et al., 2014, Newman et al., 2013). There may be variations to this rule since in hybrid aspen (*Populus tremula x tremuloides*, *Ptt*) a stoichiometry of 3:2:1 of CESA8/4/7 in developing xylem, and a stoichiometry of 8:3:1 of CESA8/4/7 was found in tension wood (Zhang et al., 2018). Wide-angle x-ray scattering analysis from the same study showed that CMF diameter was increased in tension wood, suggesting that CESA stoichiometry influences CMF properties. A recent study, showed that PttCESA8 can assemble into a catalytically active homo-oligomers *in vitro*, which may also explain the altered stoichiometry in hybrid aspen (Purushotham et al., 2020).

In addition to the CESAs, several other proteins have also been deemed essential for cellulose biosynthesis. These include CELLULOSE SYNTHASE-INTERACTING1 (CSI1), KORRIGAN1 (KOR1) and COBRA (COB). The CSCs are attached to microtubules via CSI1 and move along the cortical microtubules when depositing cellulose to the cell wall (Paredez et al., 2006, Li et al., 2012). KOR1 is an *N*-glycosylated membrane-bound endo-1,4-β-D-glucanase, which is required for cellulose biosynthesis (Nicol et al., 1998). Split-Yellow Fluorescent Protein (YFP) assays using KOR1 and either CESA1, CESA3 or CESA6, and a split-ubiquitin yeast two hybrid (Y2H) assay with KOR1 and either CESA1, CESA3 or CESA6 showed that KOR1 interacts with the primary wall CESAs (Vain et al., 2014, von Schaewen et al., 2015). Next to this, the Green Fluorescent Protein (GFP)-CESA3 movement was reduced in a *kor1-1* mutant and there was a reduced accumulation of GFP-CESA3 in the

microtubule-associated compartment after a treatment with the cellulose synthesis inhibitor CGA3259'615. These data showed that KOR1 is part of the CSC complex and suggested it is involved in the intracellular trafficking of the CSC (Vain et al., 2014). COB was first identified as a mutant with defects in anisotropic cell expansion (Benfey et al., 1993). It was later characterized to be an *N*-glycosylated, Glycosylphosphatidylinositol (GPI) plasma membrane anchored protein, which is involved in orientating of CMFs (Roudier et al., 2005). A COB-like protein Brittle Culm1 (BC1) from rice was found to be located at the plasma membrane and migrated to the cell wall after GPI-anchor cleavage (Liu et al., 2013). BC1 contains an N-terminal carbohydrate-binding module which specifically interacts with crystalline cellulose. Similarly, COBRA-LIKE2 from Arabidopsis was shown to be involved in the crystalline cellulose production of the seed mucilage (Ben-Tov et al., 2018). These results suggest that COB and COB-like proteins are involved in the synthesis of crystalline cellulose, but further research will be required to understand the underlying mechanisms.

1.2.2 Biosynthesis of hemicelluloses, pectin and glycoproteins

Hemicelluloses, pectin and the majority of glycoprotein attached glycans are synthesized/processed in the Golgi apparatus (Mohnen, 2008, Harholt et al., 2010, Scheller and Ulvskov, 2010).

The Golgi apparatus was first reported by Camillo Golgi in 1898, who initially developed a silver–osmium technique for staining nerve tissues. When observing partially silver-osmium-blackened Purkinje cells (neurons), Camillo Golgi noticed previously undescribed cytoplasmic structures. The scientific community questioned the existence of the organelle for decades, but in the 1950s with the help of a Transmission Electron Microscope (TEM) the Golgi apparatus was first photographed (Dalton and Felix, 1954).

The Golgi apparatus is located in the cytosol and composed of many small stacks of cisternae (Dupree and Sherrier, 1998). The cisternae are divided into the *cis*-Golgi, medial-Golgi and *trans*-Golgi compartments. The *cis*-golgi side receives vesicles from the ER. Vesicles move out of the Golgi apparatus at the trans-Golgi side via the Trans Golgi Network (TGN) (Gu et al., 2001). These vesicles can contain glycosylated proteins, post-translationally modified proteins/enzymes and cell wall polysaccharides, which are distributed to the cell wall, plasma membrane or to other cellular locations (Dupree and Sherrier, 1998).

The Golgi apparatus contains a highly specialized set of enzymes, which are involved in the synthesis of the different parts of the cell wall carbohydrate polymers and protein glycans. These enzymes usually contain an N-terminal

signal sequence/signal peptide comprising of 16-30 amino acids, which targets these enzymes to the Golgi membrane and orients the catalytic site into the Golgi lumen (Kapp et al., 2009). The most important group of enzymes in glycan biosynthesis are the glycosyltransferases (GTs) (Oikawa et al., 2013). GTs are able to catalyse the transfer of a glycosyl donor to a nucleophilic glycosyl acceptor molecule. In addition to GTs, also several glycosyl hydrolases (GHs) reside in the Golgi apparatus. GHs catalyse the hydrolysis of glycosidic bonds. In many cases the Golgi localized GHs are hydrolysing complex sugar chains, which upon hydrolysis become suitable glycosyl acceptor molecules for GTs (Liebminger et al., 2009). Some sugars can also be modified with methyl, acetyl and ferulic acid groups, which are attached by different transferases (Oikawa et al., 2013, Harholt et al., 2010).

Several studies have indicated that Golgi glycan synthesis happens in a sequential manner (Oikawa et al., 2013). For instance, enzymes involved in *N*-glycosylation have been found to reside in different compartments in the Golgi apparatus (Schoberer and Strasser, 2011). For example it was demonstrated that the Lewis a (Le^a) oligosaccharide Galβ(1-3)[Fucα(1-4)]GlcNAc resides in the *trans*-Golgi, based on TEM results with immunogold labelling of the Le^a specific JIM84 antibody (Fitchette et al., 1999). The terminal β-1,3-galactose on the Le^a epitope is synthesized by GALACTOSYLTRANSFERASE 1 (GALT1) in the Golgi based on GALT1-GFP and rat sialyltransferase (ST)-Red Fluorescent Protein (RFP) co-localization (Strasser et al., 2007). Based on confocal microscope imaging of known Golgi-markers, it was shown that Brefeldin A (BFA), which is an inhibitor of intracellular protein transport was able to disassemble the Golgi cisternae in a sequential manner in tobacco leaves starting from the *trans*-Golgi followed by the medial and the *cis*-Golgi (Schoberer et al., 2010). The Golgi also reassembled back in a sequential manner starting from the *cis*-Golgi when the tobacco leaves were moved to conditions without BFA. The GALT1-GFP signal disassembled earlier and reassembled later than a *cis/medial*-Golgi marker, which shows it is located in the *trans*-Golgi. In another study, the Golgi cisternae were separated using free flow electrophoresis based on differences in surface charge of the cisternae. Subsequently Golgi cisternae proteome were analysed with mass spectrometry (Parsons et al., 2019). This study confirmed that enzymes involved *N*-glycan synthesis were located in specific sub-compartments of the Golgi in agreement with the sequential synthesis of *N*-glycan structures (Strasser, 2016).

Similar results were obtained for enzymes involved in the synthesis of xyloglucan (Chevalier et al., 2010). Xylosyltransferase 1 (XT1), MURUS 3 (MUR3) and FUCOSYLTRANSFERASE 1 (FUT1) have been shown to be involved in xyloglucan synthesis (Madson et al., 2003, Edwards et al., 1999, Perrin et al., 1999). AtXT1–GFP (*cis/medial*-Golgi), AtMUR3–GFP (medial-

Golgi) and AtFUT1–GFP (*trans*-Golgi) were found to be in localized in different Golgi compartments based on immunogold labelling data imaged by TEM, which suggest that these enzymes synthesize xyloglucan in a sequential manner.

Another important factor in glycan biosynthesis is nucleotide sugar availability (Bar-Peled and O'Neill, 2011). Nucleotide sugars can be synthesized both in the cytosol and the Golgi apparatus. Usually the nucleotide sugars are synthesized in the cytosol and delivered to the Golgi by nucleotide sugar transporters (NSTs) (Knappe et al., 2003). In recent years several Golgi NSTs have been identified and characterised, but many still remain unknown (Rautengarten et al., 2014, Rautengarten et al., 2017, Saez-Aguayo et al., 2017). The recent progress in this field is facilitated by new transport assays utilising recombinant protein expression and reconstruction of the transport activity in liposomes (Rautengarten et al., 2016).

Once inside the Golgi the nucleotide sugars can be interconverted before being used for glycan biosynthesis. Uridine diphosphate glucose (UDP)-glucuronic acid decarboxylase (UXS) is an enzyme which converts UDP-glucuronic acid into UDP-Xylose. Three Arabidopsis UXS enzymes were found to be localized in the cytosol (UXS3,5,6) and three UXS enzymes were found to be localized in the Golgi apparatus (UXS1,2,4) (Kuang et al., 2016). In the *uxs3 uxs5 uxs6* triple mutant the amount of xylan was reduced, while in the *uxs1 uxs2 uxs4* triple mutant there was no obvious growth phenotype (Kuang et al., 2016, Zhong et al., 2017). Also a triple *null* mutant of Golgi localized UDP-XYLOSE TRANSPORTER1-3 (UXT1-3) caused a reduction in the amount of xylose in the cell wall (Ebert et al., 2015). These results show that nucleotide sugar transport by NSTs from the cytosol plays an important role in the supply of UDP-xylose for xylan biosynthesis in the Golgi apparatus.

UDP-D-glucose 4-epimerase 4 (UGE4) is an enzyme which interconverts UDP-D-glucose and UDP-D-galactose in *Arabidopsis thaliana* (Barber et al., 2006). UGE4 was found to localize close to the Golgi and to reside in the cytosol based on immunogold TEM and confocal microscopy data, while UGE1 and UGE2 were exclusively localized in the cytosol (Barber et al., 2006). UGE4 was found to be deficient in AGP glycosylation and β-1,4-galactan (Seifert et al., 2002, Seifert et al., 2004). All the UGE proteins were able to complement *S. cerevisiae gal10* mutant, which indicates that all of the Arabidopsis UGE proteins have the same activity (Barber et al., 2006). These results suggested that the subcellular location, and possibly tissue and/or cell type specific expression of the UGE proteins plays a functional role, and that UGE4 is in particular important in channelling UDP-galactose for GTs involved in synthesizing AGP glycosylation and RG-I glycans in the Golgi apparatus.

Thus, based on the current literature it can be concluded that the location of nucleotide sugar synthesis and nucleotide sugar transport play an important role in substrate supply to glycan synthesis and suggest that both factors can influence the amount and timing of glycan synthesis in the Golgi.

1.3 Cell expansion

Cell expansion is one of the most important and complex processes in plant development and growth. It gives rise to a great variety of cell shapes with diverse functionalities (Mathur, 2004). Cell expansion can be classified into symplastic, intrusive and protrusive growth (Guerriero et al., 2014). Symplastic growth occurs when the contact surfaces to adjacent cells are kept during cell expansion (root/shoot epidermal cells). Intrusive growth occurs when elongating cells grow in between the middle lamellas of neighbouring cells (bast fibers and xylem fibers in trees). Protrusive growth occurs when growth directs away from the surrounding plant tissues and is not limited by neighbouring cells (trichomes). Many processes play a role in cell expansion, and these will be discussed in the following paragraphs.

The cytoskeleton is an important contributor to cell expansion. The cytoskeleton is a complex, dynamic network of interlinking protein filaments, comprising of actin filaments and microtubules present in the cytoplasm of all plant cells (Mathur, 2004). The cytoskeleton is not only involved in the transport of vesicles through the secretory pathway and the movement of the CESA complex as previously discussed, but also helps to mix the cytosolic contents through so called cytosolic streaming (Verchot-Lubicz and Goldstein, 2010). Many mutants of microtubule-associated proteins (MAPs) have cell expansion defects, which manifest as changes in cell size, directional growth and twisted cells (Sedbrook and Kaloriti, 2008). Many of these defects are associated with cell wall biosynthesis. Early cell expansion mutant screens utilised Arabidopsis root growth as a readout for cell expansion defects. Among the identified mutants several were associated with cellulose biosynthesis, including COB1, POM-POM 1 (POM1) and CSI1/POM2 (Benfey et al., 1993, Hauser et al., 1995). Later POM1/2 were identified to be linkers between microtubules and the CESA complex and necessary for correct directional growth of CMFs (Roudier et al., 2005, Gu et al., 2010, Bringmann et al., 2012). These findings highlighted the importance of CMF biosynthesis and alignment for cell expansion.

Next to cellulose assembly, the primary cell wall is also modified in the apoplastic space to allow cell expansion. It is believed that acidification of the cell wall caused by auxin transport is one of the major factors in inducing cell wall loosening and expansion (Rayle and Cleland, 1992). Upon the acidification,

α-expansins are thought to be involved in loosening of the cell wall (Cosgrove, 2015). Expansins are non-enzymatic proteins found in the plant cell wall, which are able to induce cell wall creep in a pH dependent manner (Durachko and Cosgrove, 2009). This model is based on experiments where the hypocotyl cell wall of etiolated cucumber seedlings was heat inactivated to inactivate cell wall enzymes followed by extensometer analysis of the wall properties. Extensometers can be used to measure the extensibility of the cell wall under a variety of conditions. It was shown that expansins induced cell wall creep in the heat-treated cell walls of the etiolated cucumber seedlings when placed in an acidic buffer (pH < 5). Apart from expansins, xyloglucan endo-transglycosylase/hydrolase (XTH) aids in cell wall loosening (Somssich et al., 2016). XTHs can be divided into the xyloglucan endo-transglycosylases (XETs) and the xyloglucan endohydrolases (XEHs). The XETs perform non-hydrolytic cleavage and ligation of xyloglucan chains, while the XEHs results in the irreversible chain shortening of xyloglucan chains (Eklöf and Brumer, 2010). XTHs have not been show to induce cell wall loosening on their own, but did so when combined with cellulase activity (Cosgrove, 2016). This observation indicated that xyloglucan modifications alone are not enough to loosen up the cell wall and that other matrix polymers need to be modified to induce cell expansion.

Turgor pressure generated by the hydrostatic pressure within a cell pushes the cytoplasm against the cell wall providing the driving force for cell expansion (Kroeger et al., 2011). This turgor pressure can be generated by an osmotic flow, when water travels from an area with low osmotically active solutes (sugars and salts) to an area with a high amount of osmotically active solutes. Also, water transport can be facilitated by water channels, called aquaporins (Tyerman et al., 2002). These aquaporins are proteins which are embedded in membranes and are able to facilitate water transport between cells. If the pressure is strong enough and the cell wall is loose enough, the plant cell starts to expand.

To add to the complexity of cell expansion it was recently shown that the Arabidopsis pavement cells can actively shape their walls without relying on turgor pressure (Haas et al., 2020). Haas et al. (2020) showed that HG can shift between methylated and unmethylated states, which in turn shifts HG between crystalline and anisotropic phases. This results in localized swelling of the cell wall, which can drive cell expansion in pavement cells.

1.4 Cell wall model for the primary cell wall

The CMFs, xyloglucan, pectin and glycoproteins form the primary cell wall matrix, and the interaction between these polymers is one of the key features creating the unique properties of the wall. The way in which the different cell wall polymers interact and how these interactions can be modified is one of the big outstanding questions in cell wall biology. Especially the interaction of CMFs with matrix is thought to be critical for the elasticity of the wall. Early progress in this field came from the work analysing sequential extractability of the cell wall and characterization of the key molecular structures in the primary cell wall, which led to the first cell wall models (Talmadge et al., 1973, Keegstra et al., 1973, McNeil et al., 1980). Electron microscopy (EM) provided the next clue about CMF interactions with other cell wall matrix polymers, when onion cell walls were sequentially extracted and the CMFs visualised following the different extraction steps (McCann et al., 1990). Based on how the sequential extraction affected the lateral association of the CMFs it was proposed that a primary cell wall hemicellulose, most likely xyloglucan, played a role in the ordered spacing of CMFs in the primary cell wall.

More recently, the use of the atomic force microscope (AFM) has provided further insights into the cell wall organization and extensibility (Peaucelle et al., 2011). The AFM has sufficient resolution to image even individual CMFs and to measure changes in CMF and cell wall matrix organization under native conditions (Zhang et al., 2014, Zhang et al., 2016a). Besides this, the AFM in combination with an extensometer and endo-glucanase treatment has been used to simulate CMF movement/reorientation upon cell expansion (Zhang et al., 2017). The latest emerging technical advance in the field is ^{13}C multidimensional solid-state Nuclear Magnetic Resonance (NMR), which was used to analyse polymer interactions in never dried wood of spruce (Terrett et al., 2019). Such ^{13}C multidimensional solid-state NMR could potentially be used to obtain a higher resolution of the polymer interactions also in the primary cell wall.

Current primary cell wall models propose a significant role for xyloglucan in CMF - matrix interactions (Cosgrove, 2018). Xyloglucan was identified to be non-covalently interacting with cellulose and attached covalently to pectin in extracellular polysaccharides of sycamore cell suspension cultures (Bauer et al., 1973, Aspinall et al., 1969). Later, xyloglucan was found to be part of the primary cell wall of many plant species (Hayashi, 1989). For long it was believed that the cellulose/xyloglucan structure was the major load bearing structure of the primary cell wall and was essential for plant growth, but that view was challenged when an Arabidopsis mutant lacking xyloglucan was found to exhibit only minor growth phenotypes (Cavalier et al., 2008). Since then xyloglucan is rather seen as a mechanical tether that coats the hydrophobic side of CMFs

acting as one of the structural determinants of wall extensibility (Park and Cosgrove, 2015, Zhao et al., 2014, Park and Cosgrove, 2012).

Cell wall extensibility and biomechanical properties were tested in the xyloglucan deficient *xyloglucan xylosyltransferase 1* (*xxt1*)*/xxt2* mutant (Park and Cosgrove, 2012). Petioles of *xxt1/xxt2* were treated with combinations of arabinoxylan degrading enzymes and pectin degrading enzymes/chelators and cell wall creep assays showed that the *xxt1/xxt2* mutant is more responsive in the creep assay. The results suggested that both pectin and arabinoxylan have a load bearing role in the cell wall of the *xxt1/xxt2* mutant. The petioles were also treated with a mixture of proteases to assess if cell wall glycoproteins have a load bearing role in the *xxt1/xxt2* mutant. No significant change was observed in comparison to the wildtype (WT). It was hypothesized that the glycoprotein glycosylation makes the protein backbone inaccessible to the protease cocktail. Another possibility is that after protease treatment, the covalent linkage between the proteoglycans and the cell wall matrix stays intact even without the protein backbone. In the study of Tan et al. (2013) it was found that the ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN1 (APAP1) was covalently linked to arabinoxylan and pectin, and could potentially play a structural role in the wall. APAP1 is present in such a small amount in cell walls that a major load bearing function seems unlikely. However, it is possible that more AGPs are covalently bound to the cell wall in a similar way also performing load bearing functions. A set of enzymes able to break linkages between AGP glycosylation, pectin and arabinoxylan would be required to test if AGPs perform load bearing functions within the cell wall.

Pectin has also been found to associate with cellulose, but the exact nature of the interactions still needs to be elucidated. An *in vitro* study showed that sugar beet (*Beta vulgaris*) and potato (*Solanum tuberosum*) pectin may interact via neutral arabinose and galactose side chains with cellulose (Zykwinska et al., 2005). Two-Dimensional Magic-Angle-Spinning Solid-State NMR results indicated that approximately 25-50% of the cellulose comes into close proximity with pectin in the primary walls of Arabidopsis (Wang et al., 2012). Later it was shown that these type of interactions were the same between never-dried and rehydrated cell walls, which suggested that these interactions were not due to artefacts in the sample preparation (Wang et al., 2015b). The same study showed that even when 40% of the HG is extracted the cellulose-pectin interactions remain stable. This observation suggested that RG-I and a small part of the HG interact with cellulose in the Arabidopsis primary wall.

Analysis of carrot root cell walls suggested that pectin could even be covalently bound to cellulose (Broxterman and Schols, 2018). Cell walls of carrot, tomato and strawberries were sequentially extracted with increasing concentrations of

NaOH. The residue of 6M NaOH extraction, contained 31%, 11% and 5% of all galacturonic acid present in the cell wall of carrot, tomato and strawberries respectively. The residues were treated with pectin hydrolysing enzymes; a polygalacturonase or a mix of rhamnogalacturonan hydrolase, galactosidases and arabinosidases. These enzymes hardly released any products suggesting that the pectin in the samples was inaccessible to the enzymes. After a digestion with glucanases, the digestion products were first split in <10 Kilodalton (kDa) and >10Kda with a molecular cut off filter. The carrot cell wall >10kDa fraction contained pectin and 46% glucose. Since cellulose above a degree of polymerization of seven is not soluble it was suggested that the released cellulose was attached to pectin and this made it soluble. A high amount of galactose and arabinose present in this fraction, which suggested that this pectin fraction was highly branched with arabinose and galactose. It remains to be established whether the carrot cell walls are unique in this type of tight, possibly covalent, pectin to cellulose linkages.

Further support for CMF and matrix polysaccharide interactions was provided by comprehensive microarray polymer profiling (CoMPP) of sequentially extracted 2,2',2",2"'-(trans-Cyclohexane-1,2-diylbis(azanetriyl)) tetraacetic acid hydrate, CDTA (pectin), NaOH (hemicelluloses) and cadoxen (cellulose) fractions from several tissues of Arabidopsis (Moller et al., 2007). Interestingly in the cadoxen fraction, which contained most of the cellulose, also a high relative signal from antibodies recognizing β-1,4-galactan, α-1,5-arabinan, xyloglucan and AGPs was observed. These data suggest that pectin, xyloglucans and AGPs can associate with cellulose in different tissues. Since cadoxen is able to solubilize cellulose, it suggests that this association is relatively strong. Unfortunately, no RG-I specific antibodies were used, but it would be logical to find RG-I in the cadoxen fraction, since β-1,4-galactan and α-1,5-arabinans were found in this fraction.

Generating a general cell wall model based on all the known polymer structures is challenging, especially since the cell walls of many species have different compositions (Broxterman and Schols, 2018, Scheller and Ulvskov, 2010, Harholt et al., 2010). One of the most recent cell wall models based on experimental evidence, predicts that there are distinct regions within the cell wall were CMFs are closely linked to each other via xyloglucan and/or pectin (Cosgrove (2018). Cosgrove named these regions biomechanical hotspots and suggested them to be the targets of expansins and enzymes, which facilitate cell wall loosening and turgor driven cell expansion.

In my opinion too much emphasis is put on xyloglucan alone as both early and recent evidence has showed that pectin can be covalently bound to xyloglucan (Cosgrove, 2016, Aspinall et al., 1969). Evidence points to both xyloglucan and

pectin associated to cellulose have mechanical properties within the cell wall (Park and Cosgrove, 2012, Wang et al., 2012, Wang et al., 2015b, Broxterman and Schols, 2018). The data indicate that xyloglucan and pectin jointly modulate the mechanical properties of the primary cell wall. One way to test this would be to target key linkages that maintain these properties during cell wall creep assays. A mix of enzymes that target xyloglucan and key structures within RG-I could be tested. These key linkages are most likely β-1,4-galactan, α-1,5-arabinan or AGP glycan structures attached to RG-I as illustrated in Fig. 1.

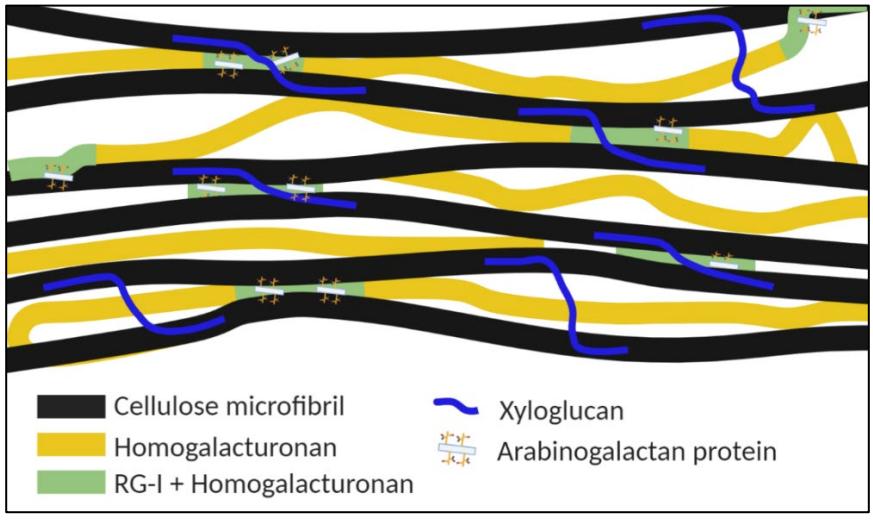

Figure 1: Proposed model of the primary cell wall
Xyloglucan coats the hydrophobic side of CMF and acts as a mechanical tether (Zhao et al., 2014). Pectin interacts with CMF, via side chains on RG-I or HG (Wang et al., 2015b). These side chains are rather short, consisting of arabinose and galactose derived from either β-1,4-galactan, α-1,5-arabinan or AGP glycan structures. Certain AGPs, like APAP1, may play structural roles in the primary cell wall and affect the interactions of xyloglucan/pectin with CMFs. CMFs, xyloglucan, pectin and AGPs come together at the proposed biomechanical hotspots (Cosgrove, 2018) and need to be modified *in muro* to allow turgor driven cell expansion.

1.5 ARABINOGALACTAN PROTEINS

AGPs are highly glycosylated cell wall proteins belonging to the HRGP group. Up to 90-98% of the total AGP mass can consist of glycans (Du et al., 1996). In the Arabidopsis genome, 85 AGPs were identified, and divided into the classical AGPs (22), Lysine rich AGPs (3), AGP peptides (16), Fasciclin-like AGPs (21) and other chimeric AGPs (23) (Showalter et al., 2010). Most AGPs contain a N-terminal signal peptide, which targets the AGP into the secretory pathway. Around 65% of the AGPs also contain a C-terminal GPI anchor, which attaches the protein to the plasma membrane. Both the signal peptide and the GPI anchor can be cleaved from the protein backbone *in vivo*. The GPI anchor cleavability could fulfil a function in relocating AGPs for signalling purposes, AGP recycling or redistributing them into the cell wall. Most identified AGP protein backbones contain amino acid repeats of Ala-Hyp, Ser-Hyp, Thr-Hyp, Val-Pro, Gly-Pro which are associated with AGP glycosylation (Showalter et al., 2010, Ellis et al., 2010). The prolines within these amino acid repeats are hydroxylated in the ER by prolyl-4-hydroxylases (P4H) (Hieta and Myllyharju, 2002). The hydroxyproline (Hyp) is then glycosylated in the ER or Golgi apparatus.

1.5.1 Enzymes involved in AGP glycosylation and the glycosylation structure

A large group of glycosyltransferases have already been identified to be involved in the glycosylation of AGPs (Table 1), but some enzymes still remain to be characterised. For instance, the *Arabidopsis thaliana* GT31 family consists of 33 members of which 20 are predicted to glycosylate AGPs (Qu et al., 2008). Eleven GT31 enzymes have been characterized so far. Of these, eight enzymes belonging to the GT31 family have been shown to be Hyp O-galactosyltransferases (table 1). These Hyp O-galactosyltransferases initiate the AGP glycosylation by adding a galactose to the Hyp in the Golgi (or possibly already in the ER). Other members of the GT31 family synthesize the β-1,3-galactan backbone of AGP glycans in the Golgi (table 1). A plethora of possible side chains are subsequently attached on the β-1,3-galactan backbone by enzymes belonging to the GT31, GT29, GT14, GT77, GT37 families (table 1, Fig. 2). Some of the glucuronic acid residues on the side chains of AGPs can possibly also be methylated (Temple et al., 2019). A rhamnosyltransferase involved in AGP glycosylation still remains to be identified. Rhamnose is potentially important in the side chains linking some AGPs to pectin (Fig. 2).

Table 1: List of characterized glycosyltransferases involved in AGP glycosylation				
	GT group	**Characterized activity**	**Cellular localization**	**Publications**
GALT1 AT1G26810	GT31	β1,3-Galactosyltransferase (N-glycans)	Golgi	(Strasser et al., 2007)
GALT2 AT4G21060	GT31	hydroxyproline-O-galactosyltransferase	ER/Golgi	(Basu et al., 2013, Basu et al., 2015b)
GALT3 AT3G06440	GT31	hydroxyproline-O-galactosyltransferase	Golgi	(Basu et al., 2015a)
GALT4 AT1G27120	GT31	hydroxyproline-O-galactosyltransferase	Golgi	(Basu et al., 2015a)
GALT5 AT1G74800	GT31	hydroxyproline-O-galactosyltransferase	Golgi	(Basu et al., 2015b)
GALT6 AT5G62620	GT31	hydroxyproline-O-galactosyltransferase	Golgi	(Basu et al., 2015a)
HPGT1 AT5G53340	GT31	hydroxyproline O-galactosyltransferase	Golgi	(Ogawa-Ohnishi and Matsubayashi, 2015)
HPGT2 AT4G32120	GT31	hydroxyproline O-galactosyltransferase	Not tested	(Ogawa-Ohnishi and Matsubayashi, 2015)
HPGT3 AT2G25300	GT31	hydroxyproline O-galactosyltransferase	Not tested	(Ogawa-Ohnishi and Matsubayashi, 2015)
GALT31A AT1G32930	GT31	β–1,6-galactosyltransferase and/or β–1,3-galactosyltransferase	Golgi	(Geshi et al., 2013, Ruprecht et al., 2020)
KNS4 AT1G33430	GT31	β–1,3-galactosyltransferase	Not tested	(Suzuki et al., 2017)
GALT29A AT1G08280	GT29	β-1,6-galactosyltransferase	Golgi	(Dilokpimol et al., 2014)
GlcAT14A AT5G39990	GT14	β –glucuronosyltransferase	Golgi	(Knoch et al., 2013)
GlcAT14B AT5G15050	GT14	β –glucuronosyltransferase	Not tested	(Dilokpimol and Geshi, 2014)
GlcAT14C AT2G37585	GT14	β –glucuronosyltransferase	Not tested	(Dilokpimol and Geshi, 2014)
GlcAT14D AT3G24040	GT14	β –glucuronosyltransferase	Golgi	(Lopez-Hernandez et al., 2020, Lao et al., 2014)
GlcAT14E AT3G15350	GT14	β –glucuronosyltransferase	Golgi	(Lopez-Hernandez et al., 2020, Lao et al., 2014)
RAY1 AT1G70630	GT77	Arabinosyltransferase	Golgi	(Gille et al., 2013)
FUT4 AT2G15390	GT37	α-1,2-Fucosyltransferases	Not tested	(Wu et al., 2010)
FUT6 AT1G14080	GT37	α-1,2-Fucosyltransferases	Golgi	(Wu et al., 2010)
FUT7 AT1G14070	GT37	α-1,2-Fucosyltransferases	Not tested	(Ruprecht et al., 2020)
AGM1 AT1G27930	DUF579	Arabinogalactan methylesterase	Golgi	(Temple et al., 2019)
AGM2 AT1G67330	DUF579	Arabinogalactan methylesterase	Not tested	(Temple et al., 2019)

The glycosylation structure of AGPs is complex, the exact structures are largely unknown and they may also vary between AGPs, tissues and cell types. AGP glycosylation is commonly depicted with a β-1,3-galactan backbone (Knoch et al., 2014, Nguema-Ona et al., 2014, Tryfona et al., 2012). Based on the binding nature of Yariv phenylglycosides (β-Yariv), it has been proposed that AGP glycans consist of a β-1,3-galactan backbone with at least seven residues (Kitazawa et al., 2013). However, NMR analysis of glycans attached to synthetic AGP motifs expressed transiently in tobacco leaves revealed a backbone of two β-1,3-galactosyl blocks, linked by a β-1,6-bond (Tan et al., 2004, Tan et al., 2010). These blocks were further decorated with galactose, arabinose, rhamnose and glucuronic acid. The cauliflower mosaic virus 35S promoter driven expression of synthetic AGP motifs may have affected the glycosylation process and hence the glycan structures may not reflect the native AGP glycans.

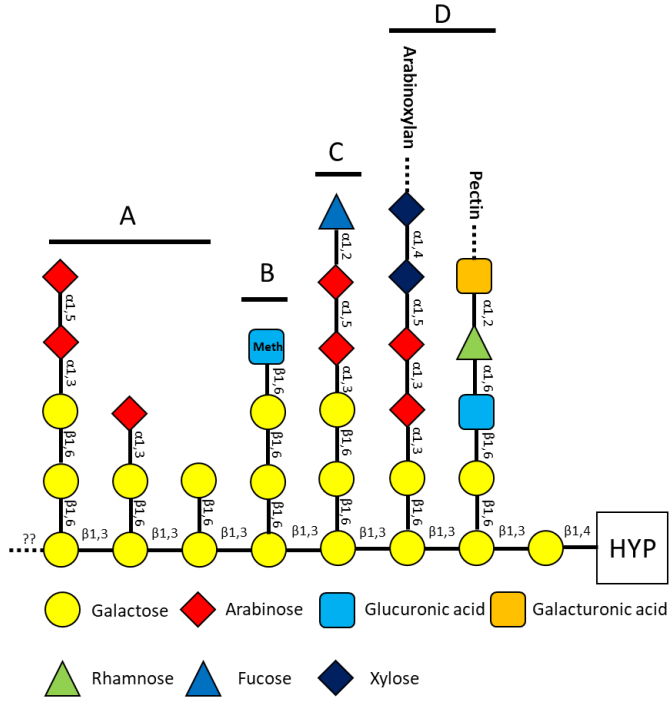

Figure 2: Summary of AGP glycosylation structures reported in literature.
(A) The most abundant mass spectrometry (MS) peaks from wheat flour AGPs digested with AGP specific hydrolases (Tryfona *et al.*, 2010). **(B)** A MS peak from Arabidopsis leaf AGPs released with AGP specific hydrolases (Tryfona *et al.*, 2012). **(C)** Fucosylated side chain in Arabidopsis WT detected with MALDI-TOF (Tryfona *et al.*, 2014). **(D)** Covalent linkages of APAP1 glycan side chains to arabinoxylan and pectin detected by 2D-NMR (Tan *et al.*, 2013)

Larger side chains, up to 14 galactose residues in length, are observed in radish roots, wheat flower, and Arabidopsis leaves based on mass spectrometric analysis of enzymatically released AGP glycans (Fig. 2) (Tryfona et al., 2010, Tryfona et al., 2012, Haque et al., 2005). It is possible that even larger side chains exist in AGPs, but these would not be detected by matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF) because of the high molecular weight. For instance, in the study of Tan et al. (2013) the AGP APAP1 was found to be covalently attached to arabinoxylan and pectin (Fig. 2). These type of side chains will not be detected on the MALDI-TOF, unless you shorten such side chains with a specific enzyme cocktail.

1.5.2 Proposed functions of AGPs and AGP glycans

The function of AGPs and AGP glycans is still highly unclear. It is thought that functional understanding of the heterogeneous AGP glycans is required to elucidate AGP roles in plants (Ellis et al., 2010). AGPs have been implicated in both signalling processes and cell wall structure, and even suggested to have a role in cellulose biosynthesis.

The evidence for AGP function in cell wall structure comes from results showing that some AGPs are associated with the cell wall matrix. So far, the only AGP which has been conclusively shown to be covalently attached to cell wall matrix is the Arabidopsis APAP1 (Tan et al., 2013). The modified sugar composition and increased extractability of pectin and xylan in 8-week-old *apap1* plants indicated that APAP1 has a structural role in the cell wall. However, the *apap1* plants were not reported to have phenotypes leaving the biological function of APAP1 unclear. Covalent attachments of AGPs to pectin have also been found in other angiosperms, like carrots (*Daucus carota*) and spent hops after beer brewing (*Humulus lupulus*) (Immerzeel et al., 2006, Oosterveld et al., 2002). The carrot and hops pectin/cell wall material came from sequentially extracted cell walls with harsh chemicals. This might have broken covalent linkages between the AGP glycans and other matrix polymers. For this reason, these data may not reflect possible native covalent interactions of AGPs in the cell wall. The role of AGPs in cell wall architecture requires further work, but based on the current knowledge it seems likely that more AGPs covalently bind to pectin, arabinoxylan or other parts of the cell wall that is currently known.

AGPs have also been proposed to act as ligands of receptors at the plasma membrane (Ellis et al., 2010). No direct evidence for this has been found so far, but there is some circumstantial evidence. It has been shown in tobacco BY-2 protoplast cells that AGP glycan epitopes co-localize with Wall Associated Kinase (WAK) at the plasma membrane (Gens et al., 2000). There is also genetic evidence for an AGP ligand function based on the analysis of quintuple mutant

of two GT31 hydroxyproline-O-galactosyltransferases, a salt oversensitive 5 (SOS5/FLA4) and two plasma membrane localized leucine-rich repeat receptor kinases (FEIs) called *galt2galt5sos5fei1fei2.* Individual *galt2galt5*, *sos5* and *fei1fei2* showed root expansion defects on sugar and salt containing media, and the quintuple mutant combining all five mutations had a similar phenotype as the individual mutants. Therefore, it was suggested that these proteins act in a single linear genetic pathway. However, further research is required to test if FEI1 and FEI2 physically interact with FLA4 at the plasma membrane, and trigger a signalling cascade.

Another proposed signalling function of AGPs is as calcium oscillators at the plasma membrane. The negatively charged glucuronic acid containing AGP glycan side chains were proposed to bind calcium and to release it in a pH dependant manner (Lamport and Varnai, 2013). It was hypothesised that rapid acidification at the periplasmic volume, caused by H+- adenylpyrophosphatase (ATPase) activity, releases calcium from AGPs. This released calcium can act as a signal, and be recycled and transported back to the plasma membrane AGP cache by exocytosis. It is plausible that AGPs can act as pH dependent calcium oscillators at the plasma membrane. Recent data, showed that a β-glucuronyltransferase triple mutant (*glcat14a/b/d*, Table 1) had reduced amounts of Glucuronic acid-containing oligosaccharides in AGP extracts (Lopez-Hernandez et al., 2020). This led to a reduced calcium binding capacity of these AGPs *in vitro,* and caused a modified fluorescence signal from the fluorescent calcium sensor R-GECO1 upon H_2O_2-induced calcium release in the roots of *glcat14a/b/e* mutant. The *glcat14a/b/e* mutant had several growth defects, including disturbed seedling growth and reduced elongation of dark-grown hypocotyls. The growth defects could be rescued by applying increased concentrations of calcium in the growth media. Another recent study provided similar evidence by showing that AGPs from mutants of GT14A-C (Table 1) had a significant reduction in calcium binding capacity in comparison to WT AGPs (Zhang et al., 2020). The simultaneous mutation of all three GT14 enzymes led to several growth defects, including delays in germination, reduced root hair length, reduced trichrome branching, defects in pollen grains, shorter siliques and reduced seed mucilage coating. These two studies show that AGPs are able to bind calcium *in vivo* and suggest that AGPs play a role in binding and releasing cell-surface apoplastic calcium, which in turn is involved in the regulation of plant growth.

1.5.3 Roles of AGPs and AGP glycans in biological processes

AGPs and AGP glycans have been associated with a multitude of biological processes. The biological functions of AGPs are unknown, but can be hypothesised based on results of *agp* mutant characterisation.

1.5.3.1 Cell expansion

AGP defects have often been linked to cell expansion. When Arabidopsis seedlings are grown on media containing 50 µM β-Yariv root cell expansion is impaired, while 50 µM α-Yariv analogue does not cause any observable root cell expansion defect (Seifert and Roberts, 2007, Basu et al., 2015b). Interestingly, the hydroxyproline *O*-galactosyltransferase *galt2*, *galt5* and *galt2galt5* mutants were less affected by 50 µM β-Yariv treatment. It was hypothesized that reduced AGP glycosylation reduces the binding of β-Yariv AGPs, which results in reduced growth inhibition. Many AGPs and AGP glycosylation mutants also show cell expansion defects. The *sos5/fla4* mutant from Arabidopsis was found to have root cell expansion defects on media containing 100 mM NaCl (Shi et al., 2003). The *agp19* mutant lacking a lysine rich AGP showed cell expansion defects, accompanied with defects in cell division, leaf development and reproduction (Yang et al., 2007). Cell expansion defects have also been observed in many mutants defect in AGP glycosylation. A double mutant of two hydroxyproline-O-galactosyltransferases, *galt2galt5*, have root cell expansion defects on media containing 100 mM NaCl (Basu et al., 2015b). As discussed earlier in section 1.5.2 it is hypothesized that GALT2 and GALT5 are involved in the glycosylation of SOS5/FLA4. A triple mutant of the hydroxyproline-O-galactosyltransferases *hydroxyproline-O-galactosyltransferase (hpgt1,2,3)* has enhanced radial cell expansion in the roots, suggesting a cell expansion defect (Ogawa-Ohnishi et al., 2013). A *fut4fut6* double mutant, lacking fucosyl units on the side chains of root AGP glycans, exhibited shorter roots on media containing 100 and 150 mM NaCl (Tryfona et al., 2014). Also, *mur1* mutants were found to have a 40% reduction in root fucose content and AGP glycosylation, which coincided with a 50% reduction in root elongation (van Hengel and Roberts, 2002). This root phenotype could be phenocopied in WT by adding eel lectin, which specifically binds to fucose in AGP glycans. These results point to a connection between AGP glycan fucose and root cell elongation. According to the current models the fucose on AGPs is linked to arabinose in the ends of the sidechains (Fig 2). The arabinosyltransferase mutant *reduced arabinose yariv 1 (ray1)* has a reduced arabinose content in β-Yariv precipitated AGPs. The *ray1* roots were shorter on nutrient agar plates and this phenotype was associated with reduced cell expansion (Gille et al., 2013). Despite these numerous examples between AGPs and cell expansion it is unclear how individual AGPs and AGP glycans affect cell expansion.

1.5.3.2 (A)biotic stress responses

AGPs have also been associated with biotic and abiotic stress responses. One of the oldest known examples is Gum Arabic, which is a complex mixture AGPs secreted from the wood in response to wounding (Whistler, 1993, Akiyama et al., 1984). Gum exudates, like Gum Arabic, are involved in sealing wounds in

the bark of trees and are also useful in many industrial applications as food stabilizers/emulsifiers/thickening agents, a binder in water colour paints, additives to ceramic glazes and more. AGPs are also secreted by some roots and act as various levels of interaction between roots and soil borne microbes on a beneficial and pathogenic level (Nguema-Ona et al., 2013). It has been suggested that AGPs are secreted from the root tips, together with pectin and other polysaccharides to either attract beneficial microbes or to encase pathogenic microbes (Driouich et al., 2013). One of the strongest pieces of evidence for AGP involvement in plant-microbe interactions comes from the characterisation of the *resistant to Agrobacterium transformation (rat1)* mutant (*RAT1* encodes for the lysine rich AGP17). *rat1* was shown to reduce the efficiency of *Agrobacterium tumefaciens* root transformation (Gaspar et al., 2004). Gaspar et al. (2004) concluded that upon *Agrobacterium tumefaciens* infection, AGP17 is required to suppress down regulation of *PATHOGENESIS-RELATED 1* (*PR1*) and to decrease the amount of salicylic acid. This leads to the suppression of the plant's defence response and allows *Agrobacterium* to infect the root.

AGPs have also been shown to have a function in abiotic stress responses, like low and high temperature tolerance, flooding, anoxia and metal deficiency/tolerance (Mareri et al., 2019). As discussed earlier, SOS5 has a root cell expansion defect on media with high NaCl, which shows that this AGP is involved in salt tolerance responses (Shi et al., 2003). The *galt2galt5* mutant showed a similar phenotype on the same media (Basu et al., 2015b). AGPs, lacking fucose side chains, also had cell expansion defects on media containing high NaCl content, which shows that AGP glycosylation is important in salt tolerance responses (Tryfona et al., 2014). AGPs are up-regulated during salt stress and it has been proposed that AGPs act as a pectin plasticizer during salt stress (Lamport et al., 2006). AGPs may decrease pectin cross linking in the cell wall matrix and in this way induce cell expansion during salt stress.

1.5.3.3 Cellulose biosynthesis

Cellulose biosynthesis appears to also require AGPs. Tension wood is a type of reaction wood formed in response to bending or leaning of the stem in angiosperm trees. Tension wood fibres form a cellulose-rich gelatinous layer (G-layer) on the lumen side (Andersson-Gunnerås et al., 2006). The G-layer can have 75-90% of cellulose (Yamamoto et al., 2005). In the study of Andersson-Gunnerås et al. (2006) it was found that some *FLA*s, *GT31* and *GT14* genes were upregulated during tension wood formation, which suggests that AGPs and AGP glycosylation play an important role in tension wood response. Another study, showed that when FLA11/FLA12 were mutated in Arabidopsis, it caused a cellulose defect in the inflorescence stem (MacMillan et al., 2010). This resulted in approximately 25% reduction in cellulose content, increased CMF angle and

altered stem biomechanics. Similar results were obtained in *Populus davidiana×Populus bolleana*, when an anti-sense transcript of a *PtFLA6* (AtFLA11/12 ortholog) was expressed under its native promoter. This resulted in reduced transcript levels of *PtFLAs* close homologs to *PtFLA6*, reductions of cellulose and lignin composition and negatively affected the stem biomechanical properties (Wang et al., 2015a). Another study showed that cellulose biosynthesis speed measured as ^{14}C glucose incorporation into the acid insoluble cell wall fraction was reduced in the *sos5, galt2galt5*, *fei1fei2* and in *sos5galt2galt5fei1fei2* root tips when plants were grown on media with 100 mM NaCl or 4.5% sucrose (Basu et al., 2016). Under these conditions the root tip in these mutants exhibits cell expansion defects. The same mutants are also hypersensitive to, and display a root expansion defects in response to the CESA inhibitor isoxaben (1-2 nM) (Basu et al., 2016).These data suggest that the cell expansion defects under these conditions are caused by a defect in cellulose biosynthesis. How FLAs affect cellulose biosynthesis is not clear, but several hypotheses have been proposed (Seifert, 2018). FLAs may bind to cellulose in the cell wall matrix, affect cellular signalling that leads to altered cellulose biosynthesis, or bind to CSCs stabilizing/altering CSC function, or they might act as adaptors between CSCs and receptor kinases to modulate CSC activity (Seifert, 2018).

1.5.3.4 Hormone signalling

Several AGP mutants have altered plant hormone content or respond differently to hormones. For example the *agp30* mutant was found to have a supressed delay of germination on abscisic acid (ABA) and an altered expression of some ABA-regulated genes, which suggested that AGP30 functions in ABA responses (van Hengel and Roberts, 2003). As earlier discussed, the *rat1* (AGP17) mutant has a reduced salicylic acid content and the amount of salicylic acid content decreases less when inoculated with *Agrobacterium* in comparison to the WT (Gaspar et al., 2004). The *sos5* and the *galt2galt5* cell expansion defect on growth media with high NaCl content can be suppressed by adding ABA or ethylene biosynthesis inhibitors (Basu et al., 2016). The study suggested that ABA and ethylene restore cellulose synthesis to WT levels, however also WT has reduced cellulose synthesis and cell expansion under these conditions. Therefore, the suppression of the cell expansion defect may simply be caused by the growth inhibitory effect of the hormones. Hence, more research will have to be performed to understand the mechanisms of ABA and ethylene on these mutants, and the relationship between AGP and hormone signalling in general.

2 Objectives

The overall aim of my PhD project was to identify glycosyl hydrolases and glycosyltransferases involved in β-1,3-galactan synthesis in the Golgi apparatus and to study their role in plant growth, cell expansion and cell wall formation in *Arabidopsis thaliana*.

The specific goals of my projects were:

I. To determine the cellular localization and to characterize the enzymatic activity of the glycoside hydrolase family 43 (GH43) proteins from *Arabidopsis thaliana* and to investigate their roles in cell expansion, cell wall formation and AGP glycan biosynthesis (Paper I).

II. To determine the cellular localization and to characterize the enzymatic activity of two glycosyltransferase family 31 (GT31) proteins from *Arabidopsis thaliana* and to investigate their roles in plant growth, cell expansion, cell wall formation, cellulose biosynthesis and AGP glycan biosynthesis (Paper II).

III. To define the phylogeny of HRGPs in aspen (*Populus tremula*), and the enzymes predicted to be involved in the HRGP glycosylation. Based on the phylogeny, analyse the gene expression profiles of the identified *HRGPs, GTs and GHs* during wood formation. And to investigate AGP/EXT glycosylation structures in phloem/cambium, developing xylem and mature xylem (Paper III).

3 Model organisms

In my studies, *Arabidopsis thaliana* (Paper I and II), *Populus tremula* and *Populus* (Paper III) were used as model organisms. Arabidopsis (*Arabidopsis thaliana*) also known as thale cress is a small flowering plant native to Europe, Asia and Africa. Arabidopsis has a rather small genome with 135 megabase pairs and it was the first plant genome that was fully sequenced (Kaul et al., 2000). Arabidopsis Transfer- Deoxyribonucleic acid (T-DNA) mutant collections consisting of random T-DNA insertions in the genome were generated in the late 1990's and early 2000's (O'Malley et al., 2015). These T-DNA collections were made available for the scientific community and greatly accelerated the *in vivo* characterization of gene function. Because of the fast generation time, small genome and available T-DNA collections Arabidopsis is an ideal model plant to study various aspects of plant growth. *Populus* sp. and *Arabidopsis* sp. are phylogenetically relatively close , which allows comparative functional studies and comparative genomics (Jansson and Douglas, 2007). The genome of *Populus trichocarpa* was the first fully sequenced tree genome, revealing an average of 1.4 to 1.6 putative *Populus* homologs for each *Arabidopsis* gene (Tuskan et al., 2006). *Populus* can be used to study various aspect of growth that cannot be addressed in Arabidopsis, like wood formation, long-term perennial growth, and seasonality. The availability of various *Populus* specific research tools, like The *Populus* Genome Integrative Explorer (POPGENIE, http://popgenie.org/) and the ASPWOOD expression database (http://aspwood.popgenie.org/aspwood-v3.0/) makes *Populus* an attractive model organism. Moreover, *Populus* can be genetically modified by *Agrobacterium* mediated transformation, which makes it possible to create mutant lines with altered gene expression using RNAi, and even loss of function mutants using gene editing tools such as the CRISPR-cas9.

4 Results and Discussion

4.1 Golgi-localized exo-beta1,3-galactosidases involved in cell expansion and root growth in Arabidopsis

At the start of this PhD project we hypothesized that besides GTs, also GHs could be involved in the synthesis of AGP glycans. The GH43 family is one of the largest known glycoside hydrolase families with over 18.000 enzymes classified as GH43 enzymes in bacteria and eukaryotes. The GH43s have been shown to have xylosidase, arabinase and galactosidase activity. The GH43 enzymes are conserved across plant kingdom, but none were characterized from plants before this project. Based on phylogeny with characterized GH43 enzymes, it was predicted that the plant GH43 enzymes possess β-1,3-galactosidase activity (Mewis et al., 2016).

4.1.1 GH43 *null* mutants are defective in root cell expansion

In paper I, we investigated the role of the two *Arabidopsis thaliana* GH43 enzymes in root cell expansion, AGP glycan synthesis and cell wall formation. We first generated GH43 single mutants (*gh43a-1, gh43b-1 and gh43b-2*) and a double mutant (*gh43null*), carrying T-DNA inserts in exons of the genes (Paper I, Fig. 1A). Several classic cell wall mutants, exhibit enhanced or conditional root growth on media containing 4.5% exogenous sugar (Benfey et al., 1993, Hauser et al., 1995). We therefore grew the *gh43* mutants on ½ media supplemented with 4.5% glucose. We showed that the *gh43null* mutant exhibits a root cell expansion defect when grown under these conditions, while having no apparent root cell expansion phenotype when grown on ½ MS media without sugar (Paper I, Fig. 1B-E). This growth defect could be complemented by introducing *GH43A-YFP* or *GH43B-YFP* driven by their native promoters into the *gh43null* mutant, which shows that the T-DNA insertions are the cause for the root cell expansion defect and that the two GH43s act redundantly. The root cell expansion defect in the *gh43null* mutant becomes visible in the root elongation zone when seedlings are placed for 10 hours on ½ MS media

supplemented with 4.5% glucose (Paper I, Fig. 2A). In line with these results the fluorescent GH43-YFP signal was high in the root elongation zone (paper I, Fig. 2B).

4.1.2 GH43A and GH43B are Golgi-localized exo- β-1,3-galactosidases

To investigate the cellular location of the GH43-YFP proteins the YFP signal was imaged with a confocal microscope. Based on initial observations both GH43-YFPs appeared to be in Golgi bodies (Paper I, Fig. 3). To confirm this, the GH43-YFP lines were crossed with the *cis*-Golgi marker SYP32-mCherry and the TGN-Golgi marker SYP43-mCherry (Uemura et al., 2004, Geldner et al., 2009). Based on co-localization with the two Golgi markers, both GH43A-YFP and GH43B-YFP were localized to the Golgi apparatus and most likely in the *cis/*medial-Golgi.

To characterize the activity of the GH43 enzymes, GH43A and GH43B were expressed in *Escherichia coli* and the recombinant proteins were purified using nickel ion affinity chromatography (Paper I, Fig. S4A). The activity of the recombinant GH43s was assayed against various of β-1,3-galactan di- and trisaccharides and the digestion products were analysed on Thin Layer Chromatography (TLC) (Paper I, Fig 4). Both GH43 proteins hydrolysed β-D-Galp-(1→3)-β-D-GalpOMe, but not β-D-Galp-(1→6)-β-D-GalpOMe. To confirm that the activity was coming from the GH43 enzymes, and not from co-purified enzymes, GH43B enzymes with mutations in predicted active sites were generated. The GH43B enzymes with the mutations, had minor or no activity versus β-D-Galp-(1→3)-β-D-GalpOMe and confirmed that the GH43B enzymes are β-1,3-galactosidases (Paper I, Fig S4C).

To search for possible native GH43 substrates in the cell wall, Arabidopsis leaf cell wall material was sequentially extracted, using 0.2M $CaCl_2$, 50 mM CDTA, 0.5M $NaCO_3$, and 4M NaOH. The GH43 enzymes did not release any sugars visible on the TLC from the sequentially extracted material (Paper I, Fig S5A). Next to this, TLC analysis did not show any released products from (partially digested) gum arabic incubated with the GH43s (Paper I, Fig S5B). These results indicated that the GH43 enzymes might be sterically hindered by side chains on β-1,3-galactan. To test this, we synthesized β-1,3-galactan oligosaccharides with a β-1,6-galactose branch on the reducing and non-reducing ends of β-D-Galp-(1→3)-β-D-GalpOMe. The GH43s were unable to hydrolyze the β-D-Galp-(1→3)-β-D-GalpOMe when the non-reducing end bore a β-1,6-galactose unit, but did hydrolyse it when the substitution was at the reducing end (Paper I, Fig 4). These results show that the GH43 enzymes are sterically hindered by β-1,6-galactan side chains and provides an explanation why nothing was visible on the TLC analysis of sequentially extracted native cell wall material and Gum Arabic. These results all together, show that the *Arabidopsis thaliana* GH43 enzymes are Golgi localized exo-β-1,3-galactosidases, and based on the prevalence of β-

1,3-galactan on AGPs, suggested that they might be involved in AGP glycan biosynthesis.

4.1.3 *gh43null* mutants have altered cell wall structure

To investigate GH43s' potential role in AGP glycan biosynthesis, we quantified the amount of $CaCl_2$ soluble and cell wall associated AGPs from 7-day-old seedlings with β-Yariv. Yariv phenyl glycosides selectively bind to the β-1,3-galactans of AGPs, enabling their spectrophotometric quantification (Kitazawa et al., 2013). β-Yariv quantification showed that the fraction of cell wall-associated AGPs in *gh43null* was higher than in the WT (Paper I, Fig 5A), while no statistically significant difference was observed for the soluble fraction (Paper I, Fig 5B). To further explore higher cell wall association of AGPs, we used a CoMPP microarray designed to determine different sugar epitopes in complex plant extracts (Moller et al., 2012). This does not only allow to assess for the extractability of AGPs, but also provides information of the extractability of cell wall matrix polymers. Sequentially extracted cell wall material with 180mM $CaCl_2$, 50 mM CDTA and 4M NaOH/26.5 mM $NaBH_4$ from 7-day-old seedlings were printed on microarrays and the CoMPP arrays were probed with monoclonal antibodies specific for pectin, xylan, xyloglucan, mannan, crystalline cellulose, extensin, AGP epitopes (Paper I, Table S2). The results show that similar to the β-Yariv data, more AGPs are found in the more tightly cell wall associated 4M NaOH/26.5 mM $NaBH_4$ fraction, which suggests that GH43 play a role in adjusting the cell wall affinity of AGPs (Paper I, Table I). Next to this, the signal of some HG/RGI epitopes were weaker in the 180mM $CaCl_2$ of *gh43null*, while the signal of some HG/RGI and xyloglucan epitopes were stronger in the 50 mM CDTA and 4M NaOH/26.5 mM $NaBH_4$. These data indicate that a change in AGP association to the cell wall, affects the extractability of other cell wall matrix polymers. In Arabidopsis, the AGP APAP1 binds covalently to RG-I/HG and was shown to increase the extractability of pectin and hemicellulosic immunoreactive epitopes, suggesting a structural cell wall cross-linker function in plant cell walls for this AGP (Tan et al., 2013). The reduced signal of the HG/RG-I specific antibodies in the $CaCl_2$ soluble fraction and the increased signal of β-1,4-galactan/HG specific antibodies in the CDTA/NaOH fractions, suggest that pectin-bound AGPs could be targets of GH43 activity.

Pectin in expanding cells has been proposed to function as a mechanical tether between CMFs (Höfte et al., 2012), and as a lubricant of CMF movement during cell expansion (Cosgrove, 2014). As described in section 1.4, pectin has been found in close proximity to CMFs and could even be covalently bound to CMFs. Based on these pectin models, AGP and pectin interactions could be expected to influence primary cell wall extensibility by restricting CMF movement and/or cell wall matrix creep. However, convincing evidence of the role of structural cell wall proteins is still wanting, and because of this they are not included in the

current cell wall models (Cosgrove, 2018). Apart from a role as structural matrix proteins, AGPs could act as matrix polymers in close proximity to CMFs and prevent other matrix polymers from binding to CMFs. This might affect the cell wall extensibility and be one of the reasons for the *gh43null* swelling phenotype. This interpretation would imply that the distribution and interaction of other polymers in the wall should also change and this seems indeed to be the case in the *gh43null* mutant based on the differential cell wall extractability.

4.2 Loss of Golgi-localized GALT7 and GALT8 reduces the rate of cellulose biosynthesis in Arabidopsis (Paper II)

4.2.1 Mutation of GALT7 and GALT8 cause primary and secondary cell wall defects

AT1G05170 (*GALT7*) and AT4G26940 (*GALT8*) are close homologs belonging to the GT31 family. Both *GALT7* and *GALT8* are co-expressed with the *GH43B* characterised in Paper I (Obayashi et al., 2007, Nibbering et al., 2020). Based on sequence similarity to characterized GT31 enzymes from *Homo sapiens*, GALT7 and GALT8 enzymes were predicted to possess β-1,3-galactosyltransferase activity (Qu et al., 2008). Both *GALT7* and *GALT8* are expressed in most tissues in Arabidopsis with the highest expression in the first internodes of the inflorescence stem (http://bar.utoronto.ca/eplant/). Because of the *GH43* co-expression and putative β-1,3-galactosyltransferase activity, I investigated the possible role of GALT7 and GALT8 in AGP glycosylation, cell expansion and cell wall formation.

To study the function of *GALT7* and *GALT8*, T-DNA insertions in their genomic sequence were isolated (Paper II, Fig. 1A). Both *galt7galt8* double mutants had apparent growth defects in seedling growth on ½ MS nutrient media plates, while the single *galt7* and *galt8* mutants resembled WT (Paper II, Fig. 1B). Cell expansion defects are common in cell wall mutants, therefore we tested for cell expansion defects in dark-grown hypocotyls. The dark-grown hypocotyls from both *galt7galt8* double mutants were ~1/3 of the length in comparison to the WT, while the single *galt7* and *galt8* mutants were not significantly different (Paper II, Fig. 1C and D). Since hypocotyl elongation is primarily driven by cell expansion rather than cell division this observation supports a function for GALT7 and GALT8 in primary cell wall biosynthesis and cell expansion. Also, 10-week-old soil grown plants of the *galt7galt8* mutants are significantly smaller at maturity than WT (Paper II, Fig. 1E). Cross sections of these plants revealed reduced fiber cell wall thickness and occasional collapsed xylem vessels (Paper II, Fig. S1 and S2). Upon closer inspection with the TEM, we confirmed the thinner secondary cell walls in both vessels, vascular fibers and interfascicular fibers of the *galt7galt8* mutants (Paper II, Fig 2). No differences were observed with the TEM in the *galt7* and *galt8* single mutants (Paper II, Supplementary

Fig. 3). To confirm that the T-DNA insertions are the cause for the growth defects, *GALT7-YFP* and *GALT8-YFP* driven by their native promoter, were transformed into the *galt7-1galt8-1* mutant. This rescued both the cell expansion defect in the dark-grown hypocotyls and the stunted growth in mature *galt7-1galt8-1* mutant (Paper II, Fig. S4 and S5). This verifies that the T-DNA insertions are the cause for the growth defects and that GALT7 and GALT8 are redundant in plant growth in *Arabidopsis thaliana*. These data together, show that GALT7 and GALT8 function redundantly in primary and secondary cell wall biosynthesis.

4.2.2 The *galt7galt8* mutants are defect in cellulose biosynthesis

To investigate the cause of the cell expansion and wall defects we analysed the cell wall composition of the mutants (Paper II, Table 1). The results show that there is a ~30% reduction in cellulose content in both the *galt7galt8* mutants, while the cellulose content in the *galt7* and *galt8* single mutants is similar to WT. The cellulose content of the *galt7galt8* mutants is also reduced in 4-day-old dark grown hypocotyls by ~30% (Paper II, Fig S6). These results show that both the primary and secondary cell wall cellulose content is affected in the *galt7galt8* mutants. The cellulose content was fully restored in the stems of the *GALT7-YFP* or *GALT8-YFP* complemented *galt7galt8* lines confirming a causal link with the cellulose defect (Paper II, Fig. S7). The monosugar and lignin content was increased in the 10-week-old inflorescence stem of both *galt7galt8* (Paper II, Table 1). Specifically, rhamnose, xylose, mannose, galacturonic acid and methylated glucuronic acid content were increased in both *galt7galt8* mutants. Since both lignin and other matrix polysaccharides are increased or at WT level as weight percentage, we hypothesized that the main defect in the *galt7galt8* mutants is in cellulose biosynthesis.

CESA proteins are essential for cellulose biosynthesis and defects/reductions in the primary and secondary cell wall CESAs, results in a reduction of cellulose biosynthesis (Taylor et al., 2003, Persson et al., 2007). Because of this, the expression and protein levels of CESA were measured. We first analysed the expression levels of the primary cell wall *CESA*s (*CESA1,3* and *6*) in 4-day-old dark grown hypocotyls and the expression of secondary cell wall *CESA*s (*CESA4,7* and *8*) in the bottom 10 cm of 20 cm long inflorescence stems. There was no difference in *CESA* expression in comparison to the WT in the *galt7galt8* mutants in either tissue (Paper II, Fig. 3A). Next, we assayed secondary cell wall CESA proteins levels using Western blots with antibodies specific for CESA4, 7 and 8 (Paper II, Fig. 3bB). The CESA4, 7 and 8 proteins levels appeared similar to WT levels in both *galt7galt8* alleles.

To further investigate the cause for the cellulose defect, we analysed for differences in cellulose synthesis rate in the *galt7-1galt8-1* mutant. *Col-0* and *galt7-1galt8-1* seedlings were supplied with ^{13}C-glucose and samples collected over a 24-hour time course. The amount of ^{13}C (%) was quantified in the total

sample (dried, ball milled powder) and crystalline cellulose. The results show that that for most time points in the total pool there is no significant difference between *Col-0* and *galt7-1galt8-1*, while there is a significant difference in the ^{13}C incorporation into crystalline cellulose pool throughout the time course (Paper II, Fig. 3C and D). These results show that there is a reduction in cellulose biosynthesis rate in the *galt7-1galt8-1* compared to WT.

4.2.3 GALT7-YFP and GALT8-YFP are Golgi localized

Both GALT7 and GALT8 are predicted to be Golgi localized. To test this, the *GALT7-YFP* and *GALT8-YFP* lines were crossed into the SYP32-mCherry (*cis*-Golgi) and SYP43-mCherry (TGN) marker backgrounds (Uemura et al., 2004, Geldner et al., 2009). Both GALT7-YFP and GALT8-YFP co-localize better with SYP32-mCherry, but do not completely overlap with SYP32-mCherry signal (Paper II, Fig. 4A). The co-localization of GALT7/8-YFP with the Golgi markers was not complete and due to a weak YFP signal it was difficult to quantify the extent of co-localization. Hence to confirm the Golgi localisation, the seedlings were treated with BFA. Treatment with BFA results in a fusion between the early Golgi cisternae and ER, while the Golgi cisternae facing the *trans* side of the Golgi and the TGN are lost to the cytoplasm and eventually to so called BFA bodies (Nebenfuhr et al., 2002). The results, demonstrate that both GALT7-YFP and GALT8-YFP co-localize better with the SYP32-mCherry marker after BFA treatment, which shows that both proteins are in the Golgi apparatus (Paper II, Fig. 4b). These results suggest that GALT7-YFP and GALT8-YFP are localized in the *cis*/medial part of the Golgi. These results are in line with the putative role of GALT7-YFP and GALT8-YFP in AGP glycosylation in the Golgi apparatus (Table I).

4.2.4 GALT8 is a galactosyltransferase

Arabidopsis GALT7 and the GALT8 belong to the GT31 clade I, which are predicted to be β-1,3-galactosyltransferases (Qu et al., 2008). Both enzymes have a predicted N-terminal transmembrane (TM) domain, suggesting Golgi membrane anchoring (Paper II, Fig. S8). The 87% amino acid sequence similarity of GALT7 and GALT8 GT domains and the redundancy observed in the phenotyping assays indicate that the proteins have the same enzymatic activity (Paper II, Fig. S8). To test for the activity a truncated version of GALT8 lacking the putative TM domain was expressed in *Escherichia coli* and the recombinant protein purified on an N-terminal GST-tag using a GST-sepharose column. The enzymatic activity of recombinant GALT8 was assayed in a reaction containing UDP-galactose as a donor and β-1,3-galactobiose as an acceptor. The reaction products were separated with TLC, which showed that the heterologous expressed GALT8 enzyme possesses galactosyltransferase activity and that GALT8 prefers pH 6.8 over pH 7 (Paper II, Fig 5).

4.2.5 *galt7galt8* mutants contain less AGP linked β-1,3-galactan

To investigate the putative roles of GALT7 and GALT8 in AGP glycosylation, the *galt7galt8* lines were examined for changes in AGP quantity and glycosylation structure. First, I quantified the amount of AGPs in 4-day-old dark grown hypocotyls, 7-day-old seedlings and the first 10 cm of the inflorescence stem with β-Yariv. The results show that in both *galt7galt8* mutants, there is a clear reduction in AGP content (Paper II, Fig. 6A). The relative change in AGP content was the most significant in the first 10 cm of the inflorescence stems (~35%) correlating with the high expression of *GALT7* and *GALT8* (http://bar.utoronto.ca/eplant/), therefore this tissue was used for further investigation. β-Yariv gel diffusion confirmed reduced AGP levels in the water-soluble fraction of the first 10 cm of the inflorescence stems (Paper II, Fig. 6B). To investigate possible changes in subpopulations of AGPs, the water-soluble AGPs were separated by gel electrophoresis and stained with β-Yariv (Paper II, Fig. 6C). This showed small shifts in the soluble AGP pool of *galt7galt8*.

To investigate whether defect cellulose biosynthesis alone could have an impact on AGP levels we quantified the β-Yariv precipitated AGPs from the inflorescence stems of the secondary cell wall *cesa4null* mutant. This assay revealed that also *cesa4null* AGP levels are significantly reduced, albeit less than in *galt7galt8*, pointing to a reciprocal relationship between AGPs and cellulose biosynthesis (Paper II, Fig. S9). Therefore, it is possible that a primary defect in cellulose biosynthesis in *galt7galt8* may exacerbate the reduction in the total AGP levels. The *hpgt1,2,3* mutant has a 90% reduction in *in vivo* hydroxyproline O-galactosyltransferase activity, accompanied with a 90% reduction in β-Yariv precipitable AGPs, while only having mild phenotypes (Ogawa-Ohnishi and Matsubayashi, 2015). The *galt7galt8* exhibits a 22-35% reduction in β-Yariv precipitable AGPs (Paper II, Fig. 6A), while exhibiting strong cell expansion defects, thinner fiber cell walls and a 30% reduction in cellulose content in inflorescence stems and seedlings (Paper II, Fig. 1, 2 and Table I). This raised the possibility that GALT7 and GALT8 act on a specific subgroup of AGPs involved in cellulose biosynthesis.

4.2.6 The protein levels of the entire FLA subgroup B are reduced in *galt7galt8*

Protein glycosylation can affect protein folding, subcellular targeting, and protein turnover (Seifert, 2020, Strasser, 2016). We hypothesized that if a subgroup of glycosylated cellulose biosynthesis related targets from GALT7 and GALT8 are mis-glycosylated, it might affect their stability and therefore their protein levels. To identify such changes, we designed a proteomics experiment to quantify differences in the relative peptide amounts from trypsin digested cell wall and membrane associated protein extracts from WT and *galt7galt8* inflorescence stems. This proteomics analysis identified 16 proteins with an

increase and 133 proteins that were decreased in both *galt7galt8* mutants at significance level P≤0.01 (Two sided student's T-test). Gene ontology (GO)-enrichment analysis, classified these identified proteins at the plasma membrane, cell wall, cytoplasm and vacuole, with roles in protein folding and stress responses (Paper II, Fig. S10). Several closely related FLA proteins were identified in the top identified proteins (Paper II, Table S2). In total 12 out of 21 FLA proteins were identified in this analysis, with significant decreases in FLA15-18 and an increase in FLA12 protein levels, while FLA1, 2, 7, 8, 10, 11 and 13 did not differ from WT (Paper II, Fig. 7). Interestingly, FLA15-18 form a subgroup (Subgroup B) in *Arabidopsis thaliana* FLA phylogeny (Johnson et al., 2003). This subgroup contains two Fasciclin-Like domains (FAS), one or two AGP glycosylation regions and lack the predicted C-terminal GPI anchor. FLA15-18 lacking the GPI anchor might mean that these proteins are not membrane anchored and are instead secreted into the apoplastic space. FLA15-18 function is unknown, but these results raise the possibility that they might be involved in cellulose biosynthesis and that GALT7 and GALT8 are involved in glycosylating this specific FLA sub group B.

We cannot exclude the possibility that GALT7 and GALT8 are involved in glycosylating CESAs or other CSC associated proteins. Yet, we did not observe a shift in CESA4,7 and 8 in the *galt7galt8* mutant line background (Paper II, Fig 3B). The possibility of CESA *N*-glycosylation has been investigated before, but considered unlikely since no molecular weight difference was observed in CESAs after PNGase-treated protein extract from the *N*-glycosylation deficient mutant *cgl1* in comparison to untreated WT CESAs (Gillmor et al., 2002). Of the known cellulose synthesis associated proteins, KOR1 and COB have been shown to be *N*-glycosylated (von Schaewen et al., 2015, Roudier et al., 2005). Both KOR1 and COB do not contain typical AGP glycosylation motifs, but unusual attached AGP glycans cannot be excluded. FLA4-citrin has been shown to contain AGP glycans, based on the binding of LM14 and JIM13 on purified FLA4-citrin on western blots (Xue et al., 2017). Even when all predicted AGP glycosylation motifs were mutated from proline to alanine, LM14 still recognized glycosylation on the purified mutated FLA4-citrin protein. Interestingly, a reduction in cellulose biosynthesis was also observed in mutants lacking the Golgi localized STELLO proteins from the GT75 family (Zhang et al., 2016b). The STELLO proteins interacted with CESAs in Y2H screens and bimolecular fluorescence complementation assays in tobacco leaf epidermis cells. The Golgi distribution, secretion and plasma membrane velocity of the CSC was impaired in the *stello* null mutant, which resulted into a reduced rate of cellulose biosynthesis. The GT75 domain was shown to be essential for STELLO function, but the enzymatic activity of the proteins was not established. Nevertheless it was hypothesized that STELLO may glycosylate CSC or associated components that impact CSC function and localization (Zhang et al., 2016b).

Based on all the results, we hypothesize that GALT7 and GALT8 are involved in glycosylating FLA15-18 and that changes in this structure affects their function. Previously, it has been shown when putative AGP glycosylation motifs were mutated from FLA4-citrin, most of its AGP glycosylation was removed, and this affected the post-secretory fate of the protein and resulted in a reduced signal of FLA4-citrin at the plasma membrane (Xue et al., 2017). Changes in FLA15-18 glycosylation in the *galt7galt8* backgrounds might have similar effects on their post-secretory fate, which in turn affects their function in cellulose biosynthesis. How FLA proteins affect cellulose biosynthesis is still left to be elucidated, but suggestions have been made. For instance, it has been proposed that FLA proteins might be involved in cellular signaling and could control cellulose biosynthesis (Seifert, 2018). Since we do not observe a significant change in CESA transcript and protein levels (Paper II, Fig 3A and B), we do not expect that the reduced cellulose biosynthesis is caused by a signaling defect, due to an unaltered transcript and protein levels of CESAs (Paper III, Fig 3A and B). The Arabidopsis *fla11/12* mutant has a reduction in cellulose and increased CMF angle, which resulted into altered stem biomechanical properties (MacMillan et al., 2010). It was proposed that FLA11 and FLA12 were involved in CMF deposition into the cell wall matrix and that this contributes to stem strength and cell wall matrix integrity. It is possible that FLA15-18 affect CMF deposition in a similar way. FLA15-18 proteins could possibly accomplish this by directly interacting with the CMF with their FAS domain and/or their glycosylation and act as a guiding mechanism for CMF into the cell wall matrix. Also, AtFLA7 and AtFLA11 orthologs have been found to directly interact with secondary cell wall CESAs in cotton fibers and secondary differentiating xylem of *Populus deltoides x trichocarpa* based on co-immunoprecipitation results with secondary cell wall CESA specific antibodies (Song et al., 2010, Li et al., 2016). FLA15-18 could interact directly with the CSC or components of the CSC and affect cellulose biosynthesis. How this could affect cellulose biosynthesis remains to be elucidated.

Another open question is how such GALT7 and GALT8 specificity for FLA15-18 would occur. GT31 enzymes from clade IV-VI contain an N-terminal galectin domain (Qu et al., 2008). This galectin domain is conserved and in *Homo sapiens* GT31 enzymes this has been characterized to act as a carbohydrate recognition domain (CRD) which has the ability to bind β-galactosides (Barondes et al., 1994, Dodd and Drickamer, 2001). This sequence is lost in GALT7 and GALT8, who instead have an N-terminal domain of unknown function 4094 (DUF 4094). This domain could potentially be involved in recognizing protein targets or specific glycosylation structures, which allows GALT7/GALT8 to perform galactosyltransferase activity on specific targets. This could explain, why only FLA15-18 protein levels are affected in the *galt7galt8* background. However, although the FLA subgroup B is a potential candidate as a target for GALT7/GALT8 activity, at this point it is also possible that other glycoprotein(s) underlie the observed phenotypes.

4.3 The distribution and function of cell wall-associated glycoproteins during wood formation in *Populus* (Paper III)

In an effort to investigate the role of HRGPs and enzymes involved HRGP glycosylation during wood formation in trees, a phylogenetic and gene expression analysis was made of the *Populus* HRGPs and the GTs and GHs that could be involved in HRGP glycosylation. In addition, immunolabeling of mature wood with AGP and EXT glycan epitope specific antibodies provided an overview of the distribution of AGPs and EXTs in wood. This data was complemented by Western blot analysis of stem tissue extracts using AGP and EXT glycan epitope specific antibodies, and β-Yariv characterization provided insights to the glycosylation structures of AGPs and EXTs in the phloem/cambium, developing xylem and mature xylem tissues.

4.3.1 The phylogeny of HRGPs and their related expression in the wood of Populus tremula

Populus trichocarpa HRGPs were identified in the study of Showalter et al. (2016) based on their typical repeating amino acid sequences, signal peptide sequence, other amino acid sequence characteristics and their similarity to already identified HRGPs. In this study, we made use of this information and HRGP nomenclature. First we obtained all of the full length protein sequences of the identified HRGPs via the POPGENIE (www.popgenie.org) database (Sjodin et al., 2009). The relative expression level values of the HRGPs from *Populus tremula* were extracted from the ASPWOOD (http://aspwood.popgenie.org/aspwood-v3.0/) database and generated wood development expression heatmaps (Sundell et al., 2017). Using these data we described the phylogeny and developing wood expression levels of the *HRGP*s in *Populus* (Paper III, Fig. 1-5).

AGPs can be subdivided into Classical AGPs, AG peptides, Lysine rich AGPs, Fasciclin-like AGPs (FLAs), plastocyanin AGPs (PAGs) and other chimeric AGPs (Showalter et al., 2016). The PAG, AG peptides and FLAs separate into different phylogenetic groups (Paper III, Fig. 1). The Classical AGPs, Lysine rich AGPs and other chimeric AGPs form more diverse mixed groups. From the identified AGPs in Showalter et al. (2016), 11 out of 27 classical AGPs, 6 out of 6 Lysine rich AGPs, 36 out of 50 FLAs, 23 out of 35 AG peptides, 22 out of 39 PAGs and 7 out of 11 other chimeric AGPs are expressed during wood formation (http://aspwood.popgenie.org/aspwood-v3.0/). We generated heatmaps displaying the relative expression of each identified AGP gene during wood formation (Paper III, Fig. 2). The well-defined expression patterns in specific wood developmental zones suggest that AGPs have specialized functions during wood formation. FLAs who are phylogenetically closely related to each other, show either high expression in the cambium/cell expansion zone, secondary cell wall formation zone or the cell wall maturation zone (Paper III, Fig. 1 and 2). The Arabidopsis ortholog AtFLA4 has been shown to be involved in cell

expansion, based on root cell expansion defects induced by NaCl stress (Shi et al., 2003). PtFLA16, 30 and 38 are close orthologs to AtFLA4 (Showalter et al., 2016) and are highly expressed in the expansion zone, which suggests that their function may be conserved between *Populus* and Arabidopsis (Paper III, Fig. 1 and 2). Similarly, many of the FLAs with high relative expression in the secondary cell wall and maturation zone are close orthologs to AtFLA11 and AtFLA12 (Showalter et al., 2016). AtFLA11/12 are highly expressed during secondary cell wall formation in Arabidopsis stems (Persson et al., 2005). These data suggest that PtFLA expression patterns and function may be conserved between Arabidopsis and *Populus*. As discussed in section 1.5.3.3 and Paper II, FLAs are predicted to be involved in cellulose biosynthesis, but the exact mechanisms are still unknown.

Most lysine rich AGPs from *Populus* are expressed in the cambial and cell expansion zone (Paper III, Fig. 2). Lysine rich AGPs from Arabidopsis have been associated with cell expansion and cell division (Ellis et al., 2010). The megaspores from the lysine rich *agp18* mutant from Arabidopsis were unable to enlarge and mitotically divide, which shows that AGP18 is essential for female gametogenesis (Acosta-García and Vielle-Calzada, 2004). Another study showed that lysine rich *agp19* mutant from Arabidopsis had fewer abaxial epidermal cells in rosette leaves, which suggests involvement in cell division (Yang et al., 2007). In addition, the shorter hypocotyl cells, smaller rosette epidermal cells and differentially shaped mesophyll cells in *agp19* were indicative of cell expansion defects. The high relative expression of the lysine rich AGPs in the wood cambial and expansion zone, suggests that they may function in cell division and/or cell expansion.

EXTs can be subdivided into Classical EXT, Short EXT, AGP/EXT hybrids and other chimeric EXT (Showalter et al., 2016). The chimeric LEUCINE-RICH REPEAT/EXTENSIN (LRX) and the PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE (PERK) and classical EXT separate into distinct groups in the phylogenetic tree (Paper III, Fig. 3). Five out of 10 LRX proteins are expressed in the wood of *Populus* (Paper III, Fig. 4). LRX are believed to be involved in cell expansion (Herger et al., 2019), which is in line with their high relative expression in the cambium and cell expansion zones. The PERKs are relatively high expressed in most wood developmental zones from *Populus* (Paper III, Fig. 4). It is interesting to note that PERKs lack the Tyrosine-X-Tyrosine motif – X being a variable amino acid - that is required for extensin cross-linking. Because of this, it is speculated that PERKs play a role in signalling, instead of being cross linked to the cell wall (Borassi et al., 2016). PERKs were proposed to be involved in the regulation of plant growth and development. Surprisingly, classical EXT do not show any measurable expression in the wood of *Populus tremula*. The Short EXT, AGP/EXT hybrids and other chimeric EXT are more mixed and either closely related to the LRX, PERK or classical EXT (Paper III, Fig. 3). The short and chimeric EXT are

expressed in most wood developmental zones and have expression in well-defined wood zones. The AGP/EXT hybrids are relatively high expressed at the end of the expansion zone and the start of the secondary cell wall formation zone, suggesting a function in the transition from primary to secondary cell wall formation.

The PRPs can be subdivided into PRPs, PRP peptide and chimeric PRPs (Showalter et al., 2016). Most of the PRP peptides can be found in a sub cluster on the phylogenetic tree (Paper III, Fig. 5). The majority of these PRP peptides are found on chromosome 17 of *Populus trichocarpa* and this most likely explains the relatedness between those PRPs (Showalter et al., 2016). Beside this, the other PRPs do not divide in specific subgroups on the phylogenetic tree, with the chimeric PRPs as the exception. The PRPs are relatively high expressed between the phloem and the expansion zone, while the chimeric PRPs are highly expressed in the phloem and cambium (Paper III, Fig. 4). In general, the PRPs relatively low expressed in the secondary cell wall formation zone and maturation zone, which suggest their main functions are in the early development of the wood.

4.3.2 The wood expression of glycosyltransferase enzymes potentially involved in AGP and EXT glycosylation in *Populus*

A broad group of glycosyltransferase enzymes are involved in the glycosylation of AGPs and EXTs. Enzymes putatively involved in AGP and EXT glycosylation in *Populus* were identified via BLAST in POPGENIE (www.popgenie.org) based on the full length protein sequence from already characterized enzymes from *Arabidopsis thaliana* (section 1.1.3, Table 1). Phylogenetic trees were generated from the *Arabidopsis thaliana* and *Populus* full length proteins sequences to identify the putative activity of these enzymes. Heatmaps were generated to show the relative expression of the genes in different wood developmental zones.

4.3.2.1 Identification of *Populus* glycosyltransferases with a putative role in AGP glycosylation

One of the most important group of glycosyltransferase enzymes involved in AGP glycosylation is the GT31 family. The GT31 family contains enzymes with characterized activity for hydroxyproline galactosyl transferase, β-1,3-galactosyltransferase and β-1,6-galactosyltransferase activity, which are both needed for AGP glycosylation (Section 1.5.1, Table 1). In *Arabidopsis* 20 GT31 family enzymes were predicted to be involved in AGP glycosylation and based on amino acid sequence similarity with characterized GT31 enzymes from *Homo sapiens* these were divided into 6 clades (Qu et al., 2008). Clade I , II and IV contain enzymes with characterized or putative β-1,3- or β-1,6-galactosyltransferase activity for AGP glycosylation based on characterised enzymes from *Arabidopsis thaliana* and cotton (*Gossypium hirsutum)* (Suzuki et al., 2017, Geshi et al., 2013, Qin et al., 2017, Ruprecht et al., 2020). Clade III,

V and VI contain characterized hydroxyproline galactosyl transferase activity for AGP protein backbone galactosylation (Ogawa-Ohnishi and Matsubayashi, 2015, Basu et al., 2013, Basu et al., 2015b, Basu et al., 2015a). AtGALT1 from clade V is the exception, with characterized β-1,3-galactosyltransferase activity for *N*-glycosylation (Strasser et al., 2007). Based on the protein sequences from *Arabidopsis thaliana*, 24 *Populus* orthologs were identified in the POPGENIE database. Orthologs were identified in all of the 6 previously described clades (Paper III, Fig. 6A). Similar to the *AGP* expression, the *GT31* genes are expressed in specific developmental zones, pointing to specific roles during wood development (Paper III, Fig. 6B). AtGALT31A from Clade II was shown to be involved in cell division of the hypophysis in Arabidopsis embryos (Geshi et al., 2013). The closest *Populus* orthologs from clade II are relatively highly expressed in the cambium, which suggest that they might fulfil similar roles in cell division during secondary growth in *Populus* (Paper III, Fig 6). Similarly, *Populus* orthologs from clade VI have a relative high expression in the cambium and wood expansion zone. AtGALT2 and AtGALT5 from clade VI have been shown to have roles in root cell expansion upon high levels of NaCl, possibly by influencing cellulose biosynthesis (Basu et al., 2016, Basu et al., 2015b). These findings suggest that at cellular level some of the *Populus* GT31s may perform similar functions than their Arabidopsis orthologs.

AGP glycan side chains are synthesised by a broad group of Golgi localized glycosyltransferases (Table 1). AtGALT29A has been characterized to possess β-1,6-galactosyltransferase activity for AGP glycosylation (Dilokpimol et al., 2014). The Arabidopsis genome contains two GT29 enzymes and based on sequence similarity, five *Populus* orthologs were identified (Kumar et al., 2019). AtGALT29 has two close *Populus* orthologs, with high relative expression in phloem, cambium and maturation zone and in the secondary cell wall formation zone (Paper III, Fig. 7). RAY1 from GT77 is so far the only glycosyltransferase characterized to have arabinosyltransferase activity for AGP glycosylation (Gille et al., 2013). The *ray1* mutants from Arabidopsis displayed shorter roots when grown on regular growth media. The two *PtRAY1* orthologs have highest expression in phloem, cambium, start of the expansion zone and the maturation zone (Paper III, Fig. 7). These data suggest that similar to their AtRAY1 ortholog, they might be involved in cell expansion in the wood. The two AtGH43s have recently been characterized as β-1,3-galactosidases localized in the Golgi apparatus (Paper I). The *gh43null* mutant exhibited a root cell expansion defect when grown on media with high sugar content, possibly through their activity on AGP glycans. The *Populus* genome contains one *PtGH43* orthologs (Kumar et al., 2019). *PtGH43* is highly expressed during secondary cell wall formation in contrast to the AtGH43, which shows highest expression during cell expansion in roots (Paper III, Fig. 7). The GT37 family from Arabidopsis consists of ten members, while the *Populus* genome only contains seven *PtGT37* orthologs (Kumar et al., 2019). Interestingly, the AtGT37 and PtGT37 protein sequences separate into distinct clades on the

phylogenetic tree. This is only seen in this GT group and could be linked to the woody perennial and herbaceous annual life styles of *Populus* and Arabidopsis, respectively (Paper III, Fig 8A). AtFUT3 is the closest AtGT37 ortholog to PtGT37 and was proposed to be a fucosyltransferase involved in cell wall biosynthesis based on overexpression associated fucose increase in the cell walls of transgenic Arabidopsis (Sarria et al., 2001). Heterologous expressed AtFUT1 has been characterized to able to transfer fucose to a specific galactose on the xyloglucan core (Cicéron et al., 2016) and is the closest homolog to AtFUT1. Next to this, AtFUT4 and AtFUT6 have been shown to act redundantly in synthesizing fucose on AGP side chains, based on hydrolysis of the root AGP backbones and identification of the released products with MALDI-TOF Mass spectrometry from the *fut4fut6* mutant (Tryfona et al., 2014). Recently, heterologous expressed AtFUT4, 6 and 7 have been characterized to be able to transfer fucose specifically on arabinofuranose residues α-1,3-linked to galactose (Ruprecht et al., 2020). Of the seven *PtGT37*s, five show expression in the wood, with unique expression profiles with a peak in either the phloem/cambium, expansion zone, secondary cell wall formation zone or maturation zone (Paper III, Fig. 8B). The GT14 family from *Arabidopsis thaliana* contains eleven enzymes, with characterized β-glucuronosyltransferase (GlcAT14) activity for glucuronic acid addition to AGP glycans from GlcAT14A-E (Knoch et al., 2013, Dilokpimol and Geshi, 2014, Lopez-Hernandez et al., 2020). The *Populus* genome contains seventeen PtGT14 orthologs (Kumar et al., 2019), with close orthologs to the characterized AtGT14 proteins (Paper III, Fig 9A). Most of the *PtGT14* genes, show high relative expression at the end of the cell expansion zone and in the secondary cell wall formation zone (Paper III, Fig. 9B). Recently, the glucuronic acid on AGP glycans was shown to be attached by AtGlcAT14 enzymes, and was shown to be important for calcium binding in a pH dependent manner (Lopez-Hernandez et al., 2020). The study suggested that such a mechanism is important for intracellular calcium signalling. Similar roles for PtGT14 members could be envisioned during wood development. However, it is likely that not all PtGT14 members are involved in AGP glycosylation. The DUF579 from Arabidopsis thaliana contains ten members, with characterized glucuronoxylan methyltransferase (GXM) activity and two characterized arabinogalactan methylesterase (AGM) enzymes responsible for methylation of the glucuronic acid on AGP glycosylation (Temple et al., 2019). Eleven *PtDUF579* orthologs are found in the *Populus* genome (Kumar et al., 2019), with two close orthologs to *AtAGM* (Paper III, Fig 10A). The two PtAGM orthologs have both a high relative expression during secondary cell wall formation, while one has a high relative expression in cambium (Paper III, Fig. 10B). The function of AGP glucuronic acid methylation is unknown, but it has been hypothesized that this methylation could potentially affect the calcium binding affinity of the glucuronic acid on AGP glycans or prevent the attachment of pectin side chains to AGP glycans (Temple et al., 2019).

4.3.2.2 Glycosyltransferase enzymes putatively involved in EXT glycosylation in *Populus*

The glycosylation of EXTs is carried out by a rather small group of enzymes in *Arabidopsis thaliana.* The serine in the EXT SP_3, SP_4 or SP_5 motifs is glycosylated by serine α-1,3-galactosyltransferase (SGT) (Saito et al., 2014). One SGT orthologs can be found in the *Populus* genome (Paper III, Fig. 11A). The hydroxyprolines of the EXT motifs are glycosylated with a β-1,4-arabinose by HPAT (Ogawa-Ohnishi et al., 2013). The *Populus* genome contains 6 HPAT orthologs (Paper III, Fig. 11A). The second β-1,2-arabinose is added by RRA (Egelund et al., 2007). Just like *Arabidopsis thaliana*, the *Populus* genome contains 3 orthologs to the RRA proteins. The second β-1,2-arabinose is further elongated with another β-1,2-arabinose by XEG113 followed by an α-1,3-arabinose by α-arabinosyltransferase (ExAD) (Gille et al., 2009, Moller et al., 2017). Both XEG113 and ExAD only have one close ortholog in the *Populus* genome (Paper III, Fig. 11A). All of the putative enzymes involved in EXT glycosylation show peak expression in specific wood developmental zones, which supports the hypothesis that EXTs have a specific function during wood development (Paper III, Fig. 11B). Arabidopsis mutants impaired in these enzymes displayed a reduction of root hair growth establishing that a glycosylation of EXTs plays an important role in tip growth of these specialized cells (Velasquez et al., 2011). Thus, it can be hypothesized that some EXTs and enzymes glycosylating EXTs may be involved in the intrusive tip growth of xylem fibers during wood formation and/or be involved in cell expansion.

4.3.3 Immunolabelling of wood with AGP and EXT glycan specific antibodies

To assess the distribution of AGP and EXT glycans in wood, cross sections of mature wood were labelled with AGP and EXT glycan specific antibodies (Paper III, Table S1). The AGP glycosylation specific antibodies LM2, LM14, MAC207, JIM8, JIM13, JIM14 and JIM16 all displayed a signal in the cell wall of xylem ray cells (Paper III, Fig. 12). The labelling of LM2, LM14 and MAC207 was rather weak (Paper III, Fig. 12A-C) in comparison to JIM8, JIM13, JIM14 and JIM16 (Paper III, Fig. 12E-F). The labelling pattern of JIM16 was distinct from the other anti-AGP antibodies and in addition to labelling xylem ray cells, JIM16 also labelled vessels (Paper III, Fig. 12G). Interestingly, the labelling of JIM16 was high in between adjacent vessel cells and in the vessel side facing xylem ray cells (Paper III, Fig. 12G). The JIM16 binds to β-1,3-galactan backbone when substituted with a single β-1,6-linked Gal residue (Ruprecht et al., 2017). The JIM16 antibody labelling data suggest that AGPs with rather short side chains show polar distribution within vessels, possibly associated with intercellular transport.

The EXT glycosylation specific antibodies LM1, JIM11, JIM12, JIM19 and JIM20 show a similar labelling pattern, forming oriented clusters in vessels towards the ray cells (Paper III, Fig. 13A-E) or neighbouring xylem vessels

(Paper III, Fig. 13F). The antibody binding pattern of all 5 EXT glycan specific antibodies, suggest that the EXT are fully *O*-glycosylated (Paper III, Table S1). The putative EXT glycosylating enzymes were relatively low expressed in the maturation zone (Paper III, Fig. 11). This may suggest that EXTs are glycosylated during active cell wall biosynthesis and that these proteins are long-lived components of the cell walls. The location of EXT epitopes overlaps with the location of pits connecting xylem vessels, and ray cells and vessels. We hypothesize that EXT may be part of this pit structure and regulate solute transport between these cells. These pits were shown to mainly contain cellulose, lignin and pectin (Herbette et al., 2015), but to our knowledge the presence of EXTs or other HRGPs have not been reported before. We speculate that EXT may play a role in the pits by participating in the modulation of pit ultrastructure and permeability. This would be in line with the previously proposed functions of EXT in the cell wall (Cannon et al., 2008, Lamport et al., 2011, Pereira et al., 2011, Chormova and Fry, 2016, Marzol et al., 2018).

4.3.3 Characterization of water soluble AGPs and EXTs by immunoblots and β-Yariv

To complement the bioinformatic and phylogenetic survey of HRGPs in the different developmental zones of aspen wood and the immunolocalization of HRGP epitopes in mature wood, we analyzed the quantity and structure of AGPs and EXTs in the water-soluble fraction from phloem/cambium, developing xylem and mature xylem.

The water soluble AGP fractions from the phloem/cambium, developing wood and mature wood were first separated by gel electrophoresis and stained with β-Yariv (Paper III, Fig. 14A). The soluble AGPs from the phloem/cambium migrated slower than the AGPs from the developing and mature wood. Proteins separate in the agarose gel, according to their size and glycosylation structures, which suggests that the AGPs from the phloem/cambium are more heavily glycosylated and/or differentially structured. The electrophoresis data also suggested that there are differences in AGP quantities between the different tissues (Paper III, Fig. 14B). To test this, the amount of AGPs were quantified from the phloem/cambium, developing xylem and mature xylem against a gum Arabic standard. The quantification shows that AGPs can be found in a higher abundance in the phloem/cambium and less in the developing and mature xylem, which is in line with the gel electrophoresis data.

During the *in situ* immunolabelling, the water soluble AGPs and the EXTs are most likely lost during the processing of the samples. To investigate the glycosylation structure of water soluble AGPs and EXTs, Western blots were performed on samples originating from the phloem/cambium, developing xylem and the mature xylem of 3-month-old hybrid aspens (*Populus tremula* x *Populus tremuloides*). The Western blots with the AGP glycosylation specific JIM8, JIM13, JIM14, JIM16, LM2, LM14 and MAC207 antibodies (Paper III, table

S1), show distinct patterns between the different tissues (Paper III, Fig. 15). In general, the antibodies bind at a higher molecular weight and at a higher intensity in the phloem/cambium in comparison to the developing and mature xylem, which is in line with the β-Yariv gel electrophoresis data. The JIM14 antibody has been characterized to bind specifically to three consecutive β-1,6-linked galactoses (Ruprecht et al., 2017). A relative weak signal is observed with the JIM14 antibody in the phloem/cambium (Paper III, Fig. 15), which suggests that these type of linkages are either not so abundant or masked by other linkages in the phloem/cambium. The LM2 antibody has been characterized to bind specifically to glucuronic acid side chains on AGP glycosylation (Smallwood et al., 1996). Even though the expression of the glucuronosyltransferase (GT14) enzymes is relatively high during secondary cell wall formation and wood maturation, the signal of LM2 is relatively weak in comparison to the phloem/cambium (Paper III, Fig. 9B and 15). The expression of AGM enzymes is relatively high during secondary cell wall formation/wood maturation and the addition of the methyl group on the glucuronic acid could potentially interfere with the LM2 binding (Paper III, Fig. 10B).

The Western blots with the EXT glycosylation specific JIM12, JIM19, JIM20 and LM1 antibodies show distinct patterns (Paper III, Fig. 15). The JIM12 and JIM20 antibodies have a strong signal in the phloem/cambium, a weak signal in the developing xylem and no visible signal in the mature xylem. Interestingly, the JIM20 antibody has a signal around 25 kDa in the phloem/cambium. Peptide EXTs with a high relative expression in the phloem/cambium have a molecular weight ranging from 10-20 kDa without glycosylation (Paper III, Fig. 4), therefore this signal could come from a low MW glycosylated peptide EXT. The JIM19 antibody has a strong signal in the developing and mature xylem, while the signal is relatively weak in the phloem/cambium. In contrast, the LM1 antibody has a strong signal in the phloem/cambium, average signal in the developing xylem and a weak signal in the mature xylem.

Altogether, both the differential binding of the AGP and EXT glycosylation specific antibodies and the β-Yariv gel electrophoresis, show that AGPs and EXT are differentially glycosylated between different tissues. This suggests that the glycosylation structure of these AGPs and EXTs play functionally important roles in wood development.

5 Conclusion and Future perspectives

In paper I, I demonstrate that the *Arabidopsis thaliana* GH43A and GH43B enzymes are involved in root growth and act redundantly during root cell expansion. Both the GH43A and GH43B enzymes are Golgi localized exo-β1,3-galactosidases. The recombinant *Escherichia coli* expressed GH43 enzymes are sterically hindered by side chains on β-1,3-galactan which is in line with their inability to release anything visible on TLC from gum Arabic and mature sequentially extracted cell wall material. The *gh43null* mutant exhibits an increase in the extent of AGP cell wall association, both demonstrated by β-Yariv quantification and CoMPP profiling of sequentially extracted cell walls. These data suggest that the GH43 enzymes are involved in AGP glycan biosynthesis in the Golgi apparatus and thereby affect root cell wall extensibility.

In paper II, I show that the GALT7 and GALT8 proteins from the GT31 family are involved in cell expansion during primary wall biosynthesis, and cellulose biosynthesis during both primary and secondary cell wall formation. Both the GALT7-YFP and GALT8-YFP are Golgi localized and most likely face towards the *cis*/medial part of the Golgi apparatus. The *galt7galt8* mutants exhibits a reduced AGP content with an altered AGP glycan structure, which is in line with their putative β1,3-galactosyl transferase activity. This is accompanied by a ~30% reduction in crystalline cellulose content and a reduction in cellulose biosynthesis rate. No significant change in the expression and protein levels of CESAs was observed, suggesting that the CESAs are not the direct cause for the cellulose biosynthesis defect. FLA15-18 protein levels are reduced in the inflorescence stems of both *galt7galt8* mutants and this could be the cause for the reduction in cellulose biosynthesis. This change in protein levels is in line with the Golgi localization and the putative β1,3 galactosyl transferase activity of GALT7 and GALT8. Further research is required to investigate FLA15-18 and their association to cellulose biosynthesis. The next step will be to characterise corresponding null mutants, and check their cellular location and glycosylation status of FLA15-FLA18 in *galt7galt8.* This could be done using FLA15-18-citrin tagged proteins in the *galt7galt8* background and compare their cellular localization in comparison to the WT with a confocal microscope. Next

to this, specific antibodies combined with gel shift assays can be used to detect any molecular weight changes.

Both the targets from GH43 (Paper I) and GALT7/GALT8 (Paper II) have yet to be identified. This could be accomplished by performing yeast two-hybrid, co-immunoprecipitation screens and TurboID-based proximity labelling to find potential interactors and substrates of these proteins. These interactors can be targeted for further investigation.

In paper III, I give an overview of the phylogeny and the relative wood expression of *HRGP*s from *Populus* and show that many of these *HRGP*s have expression patterns associated with specific wood developmental zones. This suggests that these HRGPs fulfill specific roles during wood development. Similar to these data, putative enzymes involved in HRGP glycosylation show comparable wood expression patterns, suggesting specific functions for HRGP glycosylation during wood development. These data were complemented by immunolabeling and Western blot data with AGP and EXT glycosylation specific antibodies. First, the immunolabeling shows distinct labeling patterns of the EXT and AGP antibodies in ray parenchyma cells, vessels and/or fibers. Second, the Western blots show differences in molecular weight and labelling intensity in soluble AGPs/EXTs pools from the phloem/cambium, developing xylem and the mature xylem. β-Yariv characterization of soluble AGP pools, show that the amount of AGPs and molecular weight of these AGPs is higher in the phloem/cambium in comparison to the xylem. Altogether, the data shows that HRGPs are differentially distributed and glycosylated between wood developmental zones, which suggest a specific function of AGP glycosylation. The data now opens for follow-up studies of HRGPs and glycosyltransferases associated with specific wood developmental stages.

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Popular science summary

The majority of the carbon in plant biomass is found in cell walls, which envelope all plant cells. Plant cells first build a thin and mouldable primary cell wall allowing for cell growth, which forms a basis for the huge diversity of plant shapes in nature. The majority of plant biomass accumulates in specialised cells after the cell stops expanding. These cells form a thick wall layer wall called the secondary cell wall. This secondary wall layer is strong, rigid and allows for the upright growth of land plants. Cell walls contain large polymeric molecules whose properties and interlinking gives the wall its unique properties. Some of these components are created inside the plant cell in lipid membrane enclosed factories called the Golgi apparatus. The Golgi apparatus resembles a combination of a factory assembly line and a post office. Inside the Golgi several enzymes assemble key wall components at different work stations, in the exit end of the Golgi polymers are packed and then transported to their destination in the wall. In my PhD thesis, I discovered how some of the lesser known components in the cell wall are synthesised in the Golgi apparatus. My findings contribute to the understanding of the molecular mechanisms responsible for cell wall biosynthesis and plant growth. In the future this knowledge can be used to enhance plant biomass accumulation, which is required to drive a low carbon economy.

Populärvetenskaplig sammanfattning

Huvuddelen av växters biomassa finns i cellväggen som omsluter alla växtceller. Växtceller bygger först en tunn och formbar primärcellvägg som möjliggör celltillväxt, och bidrar till den enorma mångfalden av växtformer i naturen. Majoriteten av växtbiomassan finns i specialiserade celler som bildar ett tjockt väggskikt kallad sekundärcellvägg efter att cellen slutat expandera. Detta sekundära väggskikt är starkt och stelt och möjliggör därmed upprätt tillväxt. Cellväggar innehåller polymermolekyler vars egenskaper ger väggen dess unika egenskaper. Flera av cellvägspolymererna tillverkas i den membranslutna organellen som kallas golgi-apparaten. Golgi-apparaten kan ses som en kombination av en fabriksmonteringslinje och ett postkontor. I golgi monterar enzymer väggkomponenter vid olika arbetsstationer, som sedan packas i utgångsänden av golgi, och skickas till sin destination i väggen. I min doktorsavhandling upptäckte jag enzymer som syntetiserar några av de mindre kända sockerpolymer-komponenterna i cellväggen. Mina resultat bidrar till förståelsen av de molekylära mekanismer som ansvarar för cellväggsbiosyntes och tillväxt. I framtiden kan denna kunskap användas för att öka tillväxt och växtbiomassa, vilket krävs för en hållbar bioekonomi.

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