The biology and properties of wood for nanocellulose production

Anne Bünder
Faculty of Forest Science
Department of Forest Genetics and Plant Physiology
Umeå

SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Umeå 2021

Acta Universitatis Agriculturae Sueciae
2021-73

Cover: From wood to nanocellulose
(Illustration by Daria Chrobok)

ISSN 1652-6880
ISBN (print version) 978-91-7760-823-3
ISBN (electronic version) 978-91-7760-824-0

Print: Original Tryckeri Umeå 2021

The biology and properties of wood for nanocellulose production

Abstract

Wood is a renewable and environmentally friendly raw material envisioned for the production of novel materials. In this interdisciplinary thesis project, I investigated genetic factors controlling cellulose biosynthesis, cellulose microfibril dimensions, wood mechanical properties and factors that influence nanocellulose extraction from wood.

I show that RNA-interference-mediated reduction of the CELLULOSE SYNTHASE-INTERACTIVE 1 (CSI1), which is known to link the cellulose synthase complex (CSC) to cortical microtubules (cMTs), affects wood mechanical properties as well as fibre dimensions and cellulose degree of polymerization (DP) in hybrid aspen (*Populus tremula x tremuloides).*

Furthermore, the reduced level of CSI1 was shown to negatively affect cellulose nanofibril (CNF) separation, possibly due to structural differences in cellulose microfibrils (CMFs) and/or cell wall matrix interactions. Additionally, alteration in cellulose DP and wood mechanical properties were found to be preserved in the manufactured CNF networks.

In collaboration with material scientists, I also investigated the effect of tension wood and variations in wood lignin content on nanocellulose isolation and properties. We found that the cell wall structure and composition of the tension wood, negatively affect CNF isolation when using TEMPO-mediated oxidation followed by mechanical nanofibrillation. Unexpectedly, high wood lignin content facilitated CNF isolation, potentially through increased cell wall porosity caused by TEMPO-mediated delignification. Taken together, the results show that native wood properties affect CNF isolation as well as CNF properties and motivate for further genetic improvement of trees and wood as a raw material for nanocellulose production.

Keywords: CELULOSE SYNTHASE-INTERACTING1 (CSI1), Cellulose microfibrils (CMFs), Secondary cell wall (SCW), Wood mechanical properties, Degree of polymerization (DP), Nanocellulose, Cellulose nanofibrils (CNFs), TEMPO-oxidation, Populus

Author's address: Anne Bünder, Swedish University of Agricultural Sciences, Department of Forest Genetics and Plant Physiology, SE-90187 Umeå, Sweden
Email: Anne.bunder@slu.se

The field lies open to the intellect
-Bill Mollison

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. **Anne Bünder**, Ola Sundman, Amir Mahboubi, Staffan Persson, Shawn D. Mansfield, Markus Rüggeberg, Totte Niittylä (2020). CELLULOSE SYNTHASE INTERACTING 1 is required for wood mechanics and leaf morphology. The Plant Journal, 103 (5), 1858-68.

II. Simon Jonasson, **Anne Bünder**, Oisik Das, Totte Niittylä, Kristiina Oksman (2021). Comparison of tension wood and normal wood for oxidative nanofibrillation and network characteristics. Cellulose, 28 (2), 1085-1104.

III. Simon Jonasson, **Anne Bünder**, Linn Berglund, Magnus Hertzberg, Totte Niittylä, Kristiina Oksman (2021). The effect of high lignin content on oxidative nanofibrillation of wood cell wall. nanomaterials, 11 (5), 1179.

IV. Simon Jonasson, **Anne Bünder**, Linn Berglund, Totte Niittylä, Kristiina Oksman (2021). Altered properties of cellulose nanofibrils from transgenic trees with reduced expression of *CELLULOSE SYNTHASE INTERACTING 1* (manuscript)

The contribution of Anne Bünder to the papers included in this thesis was as follows:

I. Planning and performing experiments, analyzing data, writing and formatting the manuscript

II. Performed experiments, and contributing to writing the manuscript

III. Performing chemical screen of the hybrid aspen population, analyzing data and selection of hybrid aspen lines

IV. Provided samples and data and contributing to writing the manuscript

List of figures

Abbreviations

4CL	4 COUMARATE: COENZYME A LIGASE
AFM	Atomic force microscopy
CESA	Cellulose synthase
CMF	Cellulose microfibril
cMT	Cortical microtubule
CMU	CELLULOSE-MICROTUBULE UNCOUPLING
CNC	Cellulose nanocrystal
CNF	Cellulose nanofibril
COB	COBRA
CRISPR/Cas9	Clustered regulatory interspaced short palindromic repeats/CRISPR associated
CSC	Cellulose synthase complex
CSI1	CELLULOSE SYNTHASE INTERACTIVE 1
DMAc	Dimethylacetamide
DP	Degree of polymerization
ESK1	ESKIMO1
FTIR	Fourier-transform infrared spectroscopy
G-layer	Gelatinous layer
GC/MS	Gas chromatography-mass spectrometry
IRX	IRREGULAR XYLEM
KOR	KORRIGAN
LAC	LACCASE
MFA	Microfibril angle
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NW	Normal wood

PAA	Peracetic acid
PCW	Primary cell wall
RNAi	RNA interference
SAXS	Small-angle X-ray scattering
SCW	Secondary cell wall
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
ssNMR	Solid state nuclear magnetic resonance
T-DNA	Transfer DNA
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-Tetramethylpiperidin-1-oxyl
TW	Tension wood
WAXS	Wide-angle X-ray scattering
WT	Wild type
XRD	X-ray diffraction
XXT	XYLOGLUCAN XYLOSYLTRANSFEREASE

1. Introduction: From trees to nanocellulose

Photosynthesis enables plants to use light energy to transform CO_2 and water into O_2 and carbohydrates. Thus, plants and especially trees are crucial to maintain the carbon balance on Earth. Glucose is the most abundant simple carbohydrate in nature and also building block for cellulose which in turn is considered to be the most abundant biopolymer on earth. Glucose is incorporated into long chains of cellulose, which is the main component in the plant cell wall. Cellulose in the cell wall is embedded in a matrix of hemicellulose, lignin and proteins, which together form a strong and yet flexible network. Plant cell walls provide shape and stability to the cell and allow the establishment of complex and solid structures, such as massive trees. The chemical structure of cellulose and its incorporation into the cell wall lays the foundation for one of nature's most versatile and mechanically adapted raw material: Wood. The structure of wood is highly hierarchical from different cell types in the macroscale to several cell wall layers in the microscale, to the cell wall ultrastructure and chemistry in the nano-and molecular scale (**Figure 1**). This complexity makes wood a remarkable natural biocomposite.

In a living tree, wood has three important functions: Support of the upright growth, distribution of water and nutrients and storage of nutrients and carbon. For mankind, wood and its fibres have been a renewable and versatile raw material for construction, energy production and paper manufacturing for many thousands of years. More recently wood is being used as a renewable and environmentally friendly raw material for novel products such as nanocellulose. Due to its extraordinary properties, nanocellulose can be used in new innovative biobased materials, including biocomposites, absorbents or membranes and a spectrum of new medical applications. In

this thesis I investigate wood biology and the use of aspen wood as a raw material for nanocellulose production. In the following pages I will discuss the extractability and quality of nanocellulose isolated from wood, how a better understanding of the underlying genetical mechanisms controlling cellulose biosynthesis can improve nanocellulose, and how wood structure and composition as well as wood mechanical properties can affect the extraction of nanocellulose and its final quality.

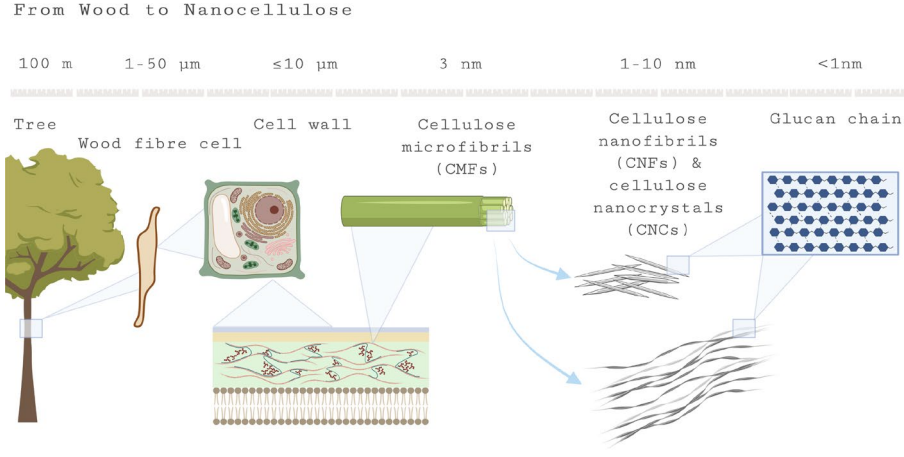

Figure 1: A scale guide from wood to nanocellulose.
Created with BioRender.com

1.1 Wood anatomy

Only 1% of the tree consists of living cells while 99% is made up of dead cells mainly in the form of wood. Wood (anatomically secondary xylem) is the water conducting tissue of the tree which also provides mechanical strength for upright growth (Spicer and Groover, 2010). The term xylem derives from the Greek word *xylon* which means wood and was introduced by the Swiss botanist Carl von Nägeli in 1858 (von Nägeli *et al.*, 1858). In a cross section of a woody stem, the innermost layer of the bark, the phloem tissue, consists of a few layers of living cells that transport photosynthetic compounds, especially sucrose from source to sink tissue. On the inside of the phloem a cylinder of meristematic cells forms the cambium that gives rise to the phloem tissue to the outside and the secondary xylem to the inside. The secondary xylem can be further divided into sapwood and heartwood. The sapwood contains a layer of newly formed xylem cells that transport sap (water, nutrients and hormones), which is pulled upwards from the roots by the transpiration stream. Eventually the innermost sapwood cells transform into heartwood which consist entirely of dead cells. In the very centre of the stem is the pith which is the oldest part of the tree and contains remnants of parenchyma cells (**Figure 2**).

The three major cell types of the secondary xylem in angiosperm trees are fibres, vessels and ray cells. These cells build up a highly organized axial and radial system of cells. Fibre and vessel cells are longitudinally elongated cells forming the axial system, whereas the ray cells are radially orientated in the stem extending from the bark to the pith. Fibre cells, with their thick cell wall, are the most abundant cells and provide support and mechanical strength to the tree. Vessel elements are comprised of several single vessel cells forming longitudinal tubes that enable xylem sap transport along the stem. Compared to the fibre cells, vessels typically have thinner cell walls which contain different types of secondary cell wall (SCW) enforcements to withstand the negative pressure generated by the transpiration stream (Harada and Côté, 1985). The radially oriented ray cells allow a radial transport of water, nutrients and photosynthates in the stem (Thomas, 1977) (**Figure 3**).

The wood anatomy of gymnosperm trees (softwood) is simpler compared to angiosperm wood. The main cell type in softwood is called tracheids (90-95%) and a minor part consists of ray parenchyma cells (5-10 %). Since softwoods lack vessel elements, the support and xylem sap transport function is shared by the tracheids (Thomas, 1977) (**Figure 3**).

The characteristic annual growth rings apparent in tree stems grown in temperate regions are formed by seasonal cues. During spring, the tree grows at a faster pace giving rise to cells with thin cell walls and wood called early wood. During the summer the tree growth slows down and the late wood with thick- walled fibre cells is formed giving rise to a denser layer of wood (Figure 3). During the transition from early wood to latewood formation, the cell diameter decreases while cell wall thickness and wood density increase. These changes in cell wall thickness and wood density were shown to be mainly driven by changes in cell size while changes in cell wall deposition rate contributes less (Cuny *et al.*, 2014).

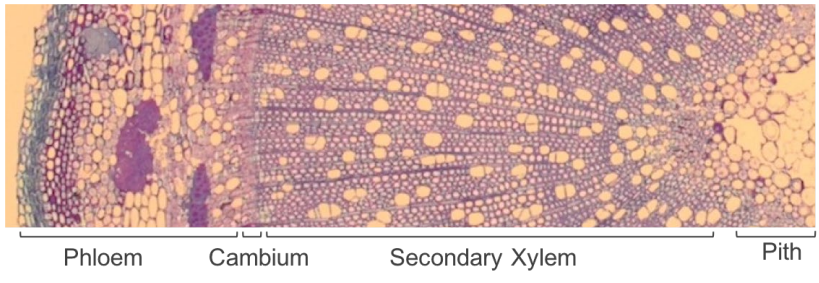

Figure 2: Cross section of a young stem from *Populus sp*.

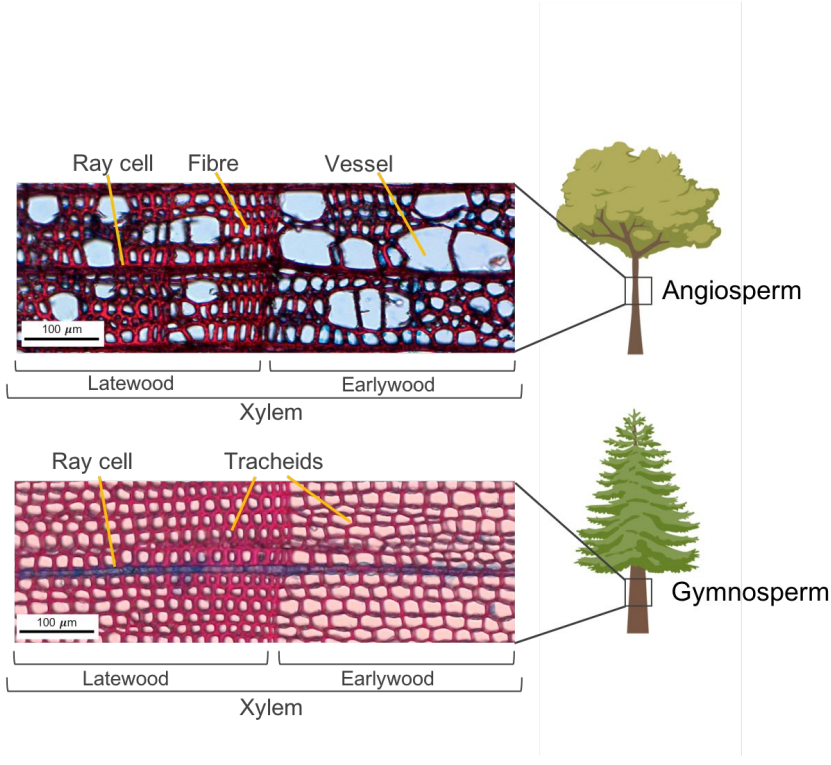

Figure 3: Angiosperm and gymnosperm wood anatomy.

1.2 Wood formation

The wood forming secondary growth allows trees to withstand wind and gravity as well as transport water to great heights. Wood formation (xylogenesis) starts in the vascular cambium which originates from the procambium, which in turn originates from the apical meristem. From the vascular cambium (secondary meristem) a single layer of bifacial cambial initials has the potential to divide and differentiate into phloem tissue outwards and secondary xylem tissue (wood) inwards. The cambial initials divide periclinal into a xylem or phloem mother cell which are almost identical to the initials. These cell types are collectively forming the cambial zone of typically several cell layers. The pluripotent cambial initials/stem cells form a single cell layer in the middle of the cambial zone. Cells of the

cambial zone produce elongated fusiform initials that give rise to the axial system (xylem and phloem) and ray initials that give rise to the radial system (ray parenchyma and ray tracheids). Phloem mother cells differentiate into phloem fibres, sieve elements, companion cells and parenchyma cells while the xylem mother cells give rise to fibres, parenchyma cells and vessel elements in the longitudinal direction. Ray initials differentiate into ray parenchyma cells elongating in the transverse direction in relation to the main growth axis (Larson, 1994, Mellerowicz *et al.*, 2001, Spicer and Groover, 2010, Larson, 2012). Xylem mother cells divide more frequently than phloem mother cells. Thus, more xylem tissue than phloem tissue is produced in most tree species, making xylem the major part of the stem biomass (Pallardy, 2010).

During the process of wood formation, the newly formed xylem cells undergo a highly regulated process of cell expansion, SCW formation and finally maturation which in the case of xylem fibres and vessels ends with programmed cell death (Mellerowicz *et al.*, 2001, Plomion *et al.*, 2001, Déjardin *et al.*, 2010, Larson, 2012). Dividing and differentiating plant cells initially form a primary cell wall (PCW) that protects the content of the cell (protoplasm) and gives shape and stability to the newly formed cell. The PCW is a thin (0.1 um), flexible and dynamic network of pectin, cellulose and hemicellulose which allows the cell to expand. During primary growth, the cells increase both in radius and in length. The expansion process can increase the volume of some xylem vessel cells by remarkable 30,000-fold in some species (Cosgrove, 2005). The final shape and dimensions of the cell is depended on turgor pressure and chemical remodelling of the PCW, cellulose microfibril (CMF) orientation as well as interaction with adjacent cells (Cosgrove, 2005). In addition, xylem fibres grow in length by intrusive tip growth, which allows the cell tip to grow in the intercellular space between neighbouring cells. This type of elongation is characteristic for fibre cells of trees and significantly contributes to the final cell length (Fahn, 1967, Gorshkova *et al.*, 2012). The final length of xylem fibres varies between tree species from for example 0.6 mm in *Populus*, 3mm in spruce and pine to remarkable 7 mm in sequoia trees (Ek *et al.*, 2009, Déjardin *et al.*, 2010).

At the end of the expansion phase, once the cell reaches its final size, a layer of secondary cell wall (SCW) is synthesized on the inner side of the cell on top of the PCW. The SCW deposition is characterized by a high rate of cellulose biosynthesis and lignification. Formation of the SCW and lignification occurs in all xylem cell types. Also, xylem ray parenchyma cells where shown to form a SCW in many tree species with a similar structure as observed in xylem fibres (Chafe and Chauret, 1974, Fujikawa, 1975, Takata *et al.*, 2019). However, not much is known about SCW formation in ray cells.

Xylem vessel elements and fibres both undergo programmed cell death, with death in vessel elements and neighbouring cells occurring slightly earlier than in fibres (Courtois-Moreau et al., 2009). Ray cells can remain alive for several years (Nakaba *et al.*, 2012). The differentiation of xylem cells is controlled by an interplay of exogenous (season, temperatures, stress) and endogenous developmental factors such as transcription factors and hormones and their interaction (Begum *et al.*, 2013, Schuetz *et al.*, 2013, Zhang *et al.*, 2014). In order to counteract gravity and heavy winds many tree species respond by fundamentally reprogramming cell wall biosynthesis and forming so called reaction wood. In angiosperm trees, the reaction wood is called tension wood (TW), which is formed at the upper side of the stem in response to gravitropic and mechanical stress and allows the stem to bend back to an upright position. In TW, an additional so-called gelatinous layer (G-layer) is often formed on top of the SCW (Dadswell and Wardrop, 1955, Mellerowicz and Sundberg, 2008, Felten and Sundberg, 2013) (**Figure. 4**). The structure and the chemical composition of the SCW is crucial for the mechanical properties of wood. The cell wall ultrastructure as well as the interaction between the three main cell wall components, cellulose, hemicellulose and lignin will be discussed in the next section.

1.3 Wood ultrastructure

The composition of cellulose, hemicellulose and lignin is relatively well understood, but how they interact and affect the cell wall structure and define the properties of the wood cell walls is not. Cellulose is the most abundant polysaccharide in the cell wall and forms cellulose microfibrils (CMFs) consisting of fibrillar crystalline aggregates of β-1,4-glucans. The CMFs are synthesized in an organized and controlled pattern contributing to the unique mechanical properties of the xylem cell walls and wood in general. In wood, the majority of the cellulose resides in the SCW of xylem cells (Li *et al.*, 2016b, Zhong *et al.*, 2019). After cellulose, the second most abundant group of polysaccharides in wood cell walls are the hemicelluloses.

Hemicelluloses are a heterogenous group of branched polysaccharides varying in their composition and structure depending on the plant species and tissue. A common feature of hemicelluloses is a backbone of ß-1,4-linked glucose, xylose or mannose. Hemicelluloses containing xylose as a backbone are often referred to xylans while mannan based hemicelluloses are simply called mannans. The major hemicellulose in angiosperm wood is glucuronoxylan while glucomannan and galactoglucomannan occur in a minor proportion. The backbone of angiosperm glucuronoxylan is made of ß-1,4-linked xylosyl residues with alpha-1,2 linked glucuronosyl and 4-O-methyl glucuronosyl side chains with partial acetylation of the xylose residues (Scheller and Ulvskov, 2010). In gymnosperm wood arabinoglucurononxylan, glucomannan and galactoglucomannan are the most prominent hemicelluloses (Timell, 1967, Scheller and Ulvskov, 2010). (Galacto)glucomannan, which is the most common hemicellulose in gymnosperm trees is built up of a ß-1,4-linked mannopyranose and glucopyranose backbone with alpha-1,6 linked galactopyranose side chains and irregularly substituted by O-acetyl groups (Sjöström, 1993). Depending on the galactose content, (galacto)glucomannan is either referred to glucomannan or galactoglucomannan.

The diverse structure of hemicelluloses is owned to the type and distribution of the side chains attached to the backbone and differ between cell types and plant species (Scheller and Ulvskov, 2010). The diverse chemical composition and structure as well as spatial distribution of the side chains were shown to affect the binding capacity to cellulose and thus to the overall

cell wall properties (Grantham *et al.*, 2017). In the G-layer of TW, galactan with a backbone of ß-1,4-linked galactopyranose which is highly substituted with complex side chains mainly containing galactose but also glucuronic acid, galacturonic acid, rhamnose and arabinofuranosyl residues, was found to be the dominant hemicellulose (Shimizu 2001). Galactan is hypothesized to be involved in the tensional stress generation, while being entrapped in between the CMFs in the G-layer (Gorshkova *et al.*, 2015).

Lignin introduces stiffness and strength to the cell wall, makes the cell wall more hydrophobic and its complex and compact structure also protects the woody tissues against microbial degradation (Vanholme *et al.*, 2010, Miedes *et al.*, 2014). Additionally, lignin in wood helps to attach the cells to each other as it is a major component of the middle lamella adjoining neighbouring cells (Zamil and Geitmann, 2017). Lignin is a hydrophobic polymer forming a highly complex 3-dimensional network of aromatic and aliphatic moieties covalently linked via ether (C-O-C) and carbon-carbon (C-C) bonds. The lignin network is mainly built up of three monomers, termed monolignols. These are p-coumaryl alcohol (H-lignin), coniferyl alcohol (G-lignin) and sinapyl alcohol (S-lignin). The monolignols differ in the number of methoxy groups attached to the aromatic ring. While p-coumaryl alcohols do not have any methoxy groups, coniferyl-alcohols have one methoxy group at the 3-position and sinapyl alcohols have two methoxy groups at the 3- and 5-position of the aromatic ring. Lignin polymerization occurs via radical coupling forming covalent bonds between the monolignols. Depending on the plant species and even cell type, the ratio between these monomers in the lignin complex varies. While grasses contain all three monolignols (S-, G- and H-lignin), softwood lignin (gymnosperms) consist mainly of coniferyl alcohol (G-lignin) with small amounts of p-coumaryl alcohol (H-lignin). Hardwoods (angiosperms) contain both sinapyl and coniferyl alcohol (S- and G-lignin) with traces of p-coumaryl alcohol (H-lignin) in some species (Vanholme *et al.*, 2010, Ye and Zhong, 2015, Zhong *et al.*, 2019). The lignin network structure depends on the composition and ratio of the individual monolignols and their chemical structure. The methoxy group at the 5-position of sinapyl alcohols hinders a radical coupling at the 5-position. Thus, due to the higher proportion of sinapyl alcohol and its structural features, it is assumed that hardwood lignin is more linear and less branched compared to softwood lignin. The proportions of coniferyl and sinapyl

alcohol were assumed to affect the degree of crosslinking of the polymer and thus the mechanical properties of the wood. The occurrence of sinapyl alcohol in angiosperms is assumed to make the lignin structure and thus the wood more flexible but still strong (Bonawitz and Chapple, 2010).

1.3.1 Primary cell wall

The primary cell wall is a dynamic structure composed of pectins (30-50 %), cellulose (15-40 %), hemicellulose (20-30 %) and up to 10 % of structural proteins, cellulose being the main load bearing polymer in the PCW (Anderson *et al.*, 2010, Cosgrove, 2012). The composition and interaction of the PCW matrix is important for the elasticity of the cell wall allowing it to expand and withstand turgor pressure (Cosgrove, 2005). For long time the cellulose-xyloglucan structure and their interaction were considered to play an essential role in cell expansion and biomechanics of the PCW and thus plant growth. This assumption was based on the model in which xyloglucan coats the hydrophobic side of the CMFs and thus separates individual CMFs and prevents contact between the individual CMFs (Carpita and Gibeaut, 1993, Somerville *et al.*, 2004). However, this model was challenged by Arabidopsis mutants *(xxt1/xxt2*) lacking xyloglucan. Unexpectedly, these mutants only showed a minor growth phenotype. These results indicated that xyloglucans are of lesser significance for PCW expansion and plant growth than earlier assumed, but potentially act as a mechanical and structural tether during PCW expansion (Cavalier *et al.*, 2008). The latest cell wall model(s) suggests that xyloglucan only links at distinct regions to the CMFs rather than coating the entire CMF surface (Park and Cosgrove, 2015). Recent solid-state NMR (ssNMR) analysis of the PCW revealed that approx. 50 % of the cellulose surface is in contact with pectin, but only a minor part of the xyloglucans interacts with cellulose (Wang et al 2012). These results led to the assumption that individual CMFs in the PCW are separated by pectin rather than xyloglucan (Dick Perez et al. 2012). Hence, current models assume that both xyloglucan and pectin are associated to cellulose and contribute to the mechanical properties of the PCW (Park and Cosgrove, 2012, Park and Cosgrove, 2015, Broxterman and Schols, 2018).

1.3.2 Secondary cell wall

The secondary cell wall accounts for more than 80% of the fibre wall weight and thus contributes most to the total woody biomass (Fengel and Stoll, 1973, Sorieul *et al.*, 2016). The composition and the interaction between the cell wall polymers in the SCW are fundamental for the mechanical properties of the individual xylem cell and wood as a whole. The SCW of xylem fibres are composed of cellulose (40-80%), hemicellulose (10-40 %) and lignin (5-25 %) with variations depending on cell type, tissue and plant species (Kumar *et al.*, 2016). By using polarized light or electron microscopy, the thick SCW typically shows a separation into three individual SCW-layers (S1, S2, and S3) (Bailey and Kerr, 1935, Wardrop and Preston, 1947). The S1 layer is the transitional layer between PCW and SCW and has typically a thickness of 0.1-4 µm. The S2 is the thickest layer with 1-10 µm and contributes most to the woody biomass and properties of the cell wall. The S3 layer, the innermost layer, is typically 0.5-1 µm thick (Plomion *et al.*, 2001). In TW, xylem fibre cells form a so-called gelatinous layer (G-layer) often overlaying the S2 and replacing the S3 layer (Dadswell and Wardrop, 1955, Norberg and Meier, 1966) (**Figure 4).** The G-layer contains almost pure crystalline cellulose and a minor amount of matrix polymers (Nishikubo *et al.*, 2007).

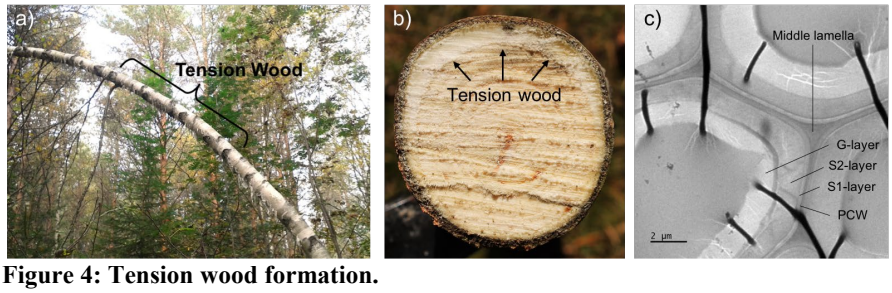

Figure 4: Tension wood formation.
a) Leaning birch (*Betula pubescens*) tree. b) Cross section of a stem with tension wood (lighter coloured) on the upper side. c) Electron micrograph of tension wood fibre cross section with the different cell wall layers indicated.

Already in 1935, Bailey and Kerr observed the individual cell wall layers of the SCW in gymnosperm tracheids and angiosperm fibres by using polarized light microscopy. This technique is based on the principle of birefringence. This means that crystalline cellulose rotates, and splits polarized light in very thin wood sections. This allows polarized light microscopy to visualize and

differentiate between the S-layers due to changes in their refractive properties (Bailey and Kerr, 1935). These differences can be seen in changed brightness between the wall layers which results from different orientations of the polarized light after it passes through the wall (Bailey and Kerr, 1935, Donaldson, 2008, Abraham and Elbaum, 2013). Hence, these early observations by Bailey and Kerr indicated structural differences between the layers caused by the orientation of CMFs. In addition, it was assumed that chemical variation occurs between the different S-layers (Bailey and Kerr, 1935). The early polarized light microscopy measurements were later confirmed by using electron microscopy techniques where the three SCW layers were distinguished by their difference in molecular structure and composition apparent as different brightness in the micrographs. These differences were mainly explained by the altering angle of which CMFs are deposited into the cell wall (Wardrop and Preston, 1947, Hodge and Wardrop, 1950, Abe *et al.*, 1991, Brändström *et al.*, 2003). The orientation of the CMFs in the SCW is referred as the cellulose microfibril angle (MFA) and is defined by the angle between the helical arrangement of CMF and the longitudinal fibre axis (Barnett and Bonham, 2004). That means that CMFs that are aligned more parallel to the fibre axis have a lower MFA.

A wide range of methods including electron microscopy, X-ray diffraction (XRD) and near infrared (NIR) spectroscopy, supports the current cell wall model in which the clear visual separation into individual SCW layers is defined by the MFA (for review see Donaldson (2008)). Currently, the most common and reliable method to measure MFA is by using XRD including wide angle X-ray scattering (WAXS) and small angle X-ray scattering (SAXS) (Cave, 1997, Evans, 1999, Sarén and Serimaa, 2006). In the S1 and S3 layer of xylem fibres, the CMFs were measured to orient close to perpendicular to the cell axis resulting in a high MFA compared to the angle in the S2 layer which is, due to the almost axial orientation of the CMFs, much smaller. Typically, CMFs are measured to orient in an angle of 60-80° in the S1 layer. The S2 layer is reported to have a cellulose MFA of 5-30 ° and the S3 layer a MFA of 60-90° (Plomion *et al.*, 2001). In the G-layer of tension wood fibres from poplar trees the MFA was measured to be 0-4° (Goswami *et al.*, 2008, Lautner *et al.*, 2012, Rüggeberg *et al.*, 2013). However, even XRD measurements have their limitations and are restricted by the beam size used. Thus, it often can only give an average of the MFA

of all three SCW layers. In most models of xylem fibre walls the MFA change between the S-layers is depicted as a sharp boundary. However, this may not be true. A study by Abe *et al*. (1991) using field emission scanning electron microscopy (FE-SEM) suggested a more gradual change of the MFA between the S1 and S2 layer and the existence of a transitional layer between the S1-S2 layer and S2-S3 layer was proposed (Abe *et al.*, 1991). More recently, Reza *et al*. (2019) observed the three-dimensional (3D) structure of the transitional layer between the S1 and S2 layer by applying single-axis tomography on ultrathin spruce wood sections. These results suggested that a fast change in the MFA occurs in the transitional S1-S2 layer followed by a more gradual change within the S2 layer. Additionally, the transitional layer was considered not only to differ in its structure from the neighbouring layer, but also in density and potentially in its chemical composition (Bailey and Kerr, 1935, Reza *et al.*, 2019).

Microscopy can also provide information about compositional differences between the different S-layers by using specific staining or immunolabelling methods. Immunolocalization and fluorescence microscopy experiments have provided support for a different composition between the individual SCW layers in pine tracheids. Stronger signals of lignin were detected in the S1 and S3 compared to the S2 layer while galactoglucomannan was more dominant in the S2 layer (Donaldson and Knox, 2012). Another study using immunofluorescence and immune gold labelling showed a higher proportion of mannans in the S2 layer in poplar SCW compared to S1 and S3 while xylans were more homogeneously distributed among the SCW (Kaneda *et al.*, 2010). Hence, it can be concluded that the individual SCW layers differ from each other in both structure and chemical composition. These results imply that both MFA and cellulose microfibril matrix interactions as well as chemical composition can influence the ultrastructure of wood and its mechanical properties.

1.3.3 Cellulose-matrix polymer interactions

Hemicellulose

The elucidation of molecular interactions between CMFs and hemicelluloses *in situ* has been technically challenging. Already in the early 90s, based on XRD, hemicelluloses in SCW were proposed to cross-link with CMFs facilitating aggregation into higher structures (Atalla *et al.*, 1993). Convincing evidence of covalent links between CMFs and hemicelluloses is lacking and the type and degree of interaction between cellulose and hemicellulose is still a subject of debate. Recent improvements in solid state nuclear magnetic resonance (ssNMR) have provided new insights into the interactions between cellulose and the other matrix polymers (Simmons *et al.*, 2016, Grantham *et al.*, 2017, Kang *et al.*, 2019).

Using ssNMR techniques, intermolecular interactions between cellulose and xylans were recently studied in cell walls of Arabidopsis. Two-fold helical screwed xylans (one 360° twist per two glycosidic bonds) were shown to associate with cellulose via hydrogen bonds. In solution, xylan exhibits a three-fold helical screwed structure (one 360° twist per three glycosidic bonds) but adopts a two-fold screw conformation when it binds to the hydrophilic surface of cellulose (Nieduszynski and Marchessault, 1971, Preston, 1979, Simmons *et al.*, 2016). Simulations predicted that xylan would adopt a two-fold screw conformation when interacting with cellulose given that xylan chains are evenly substituted. This xylan conformation is required to bind onto the cellulose surface (Busse-Wicher *et al.*, 2014). Additionally, using cryo-scanning electron microscopy (cryo-SEM), it was shown that in Arabidopsis mutants having reduced amounts of xylan (*irx9* and *irx 10*) and reduced xylan-acetylation (*esk1*) the median macrofibril diameter was significantly reduced by 10-30% compared to the WT (Lyczakowski *et al.*, 2019).

The interactions between cellulose and xylan were also shown to affect the MFA in aspen wood. A suppression of xylan endotransglycosylase resulted in a decrease in the cellulose MFA in the S2 layer of hybrid aspen (*Populus tremula x tremuloides*) implying a role for xylan mediated matrix effects in orienting CMFs (Derba-Maceluch *et al.*, 2015). This emphasises the

complexity of interpreting results of mutant studies, and that matrix effects can influence cell wall polymer biosynthesis as well as interactions between polymers. Nevertheless, these findings indicated that in the SCW xylan coats the surface of the CMFs and affects their diameter as well as making the hydrophilic cellulose surface more hydrophobic (due to acetate groups) and acidic (due to glucuronic acid groups).

Lignin

Early computational simulations based on models of lignin interactions in the SCW proposed that cellulose is cross-linked with lignin (Houtman and Atalla, 1995). However, a recent study by Kang et al (2019) using solid state NMR spectroscopy (ssNMR) showed that, in the SCW of maize stems at least, lignin mainly binds xylan. It was shown that lignin methyl ethers predominantly bind to the polar motifs of 3-fold xylans (one 360° twist per three glycosidic bonds) via electrostatic interactions, which are not closely associated with cellulose due to steric hindrance. These results suggest a model in which xylan spaces in between and adjoins lignin and cellulose (Kang *et al.*, 2019). The ssNMR technique has provided highly valuable insights into the molecular interactions between lignin and polysaccharides but the assembly into higher order structures is still debated.

In a recent study by Lyczakowski *et al.* (2019) using cryo-SEM it could be shown that not only xylan contributes to the median diameter of cellulose macrofibrils but also lignin. In Arabidopsis mutants with reduced amounts of lignin (*4cl and lac4*) the median cellulose macrofibril was observed to be significantly reduced by 15% and 7% respectively. These results suggest that in the native state, bundles of cellulose macrofibrils are composed of cellulose, xylan and lignin in the SCW of Arabidopsis (Lyczakowski *et al.*, 2019). The most recent model of cell wall matrix interactions suggests that in the SCW CMFs are mainly linked to and connected via xylan while lignin predominantly links to three-fold xylan via electrostatic bonds. Thus, xylan is assumed to adjoin lignin and cellulose (Kang *et al.*, 2019). **Figure 5** below summarises our current understanding of the cell wall polymer interactions in the SCW.

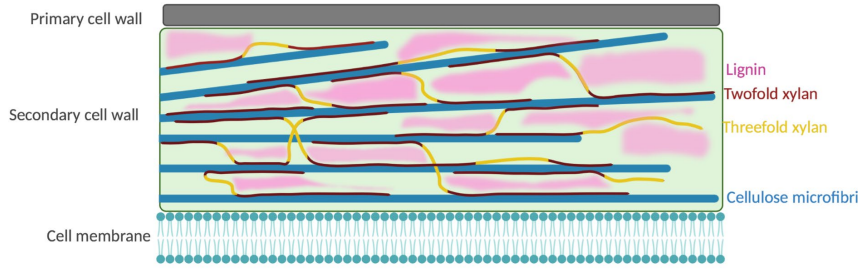

Figure 5: **Model of the secondary cell wall matrix interactions.**
Cellulose microfibrils (CMFs) (blue) bind to two-fold xylan (red) while lignin (pink) preferentially binds to three-fold xylan via electrostatic bonds. Xylan links and adjoins cellulose and lignin. This model is adapted from Kang *et al.* (2019). Created with BioRender.

Analysis of isolated cell wall components is important for understanding their chemical structure and nature but does not explain how they perform in the network of the cell wall and thus contribute to its mechanical properties. It is of importance to conceive the cell wall structure as a whole including the different components, their individual chemical structure and architecture as well as the interactions with each other in order to understand the effect on the overall wood properties. Cellulose is considered as the main load bearing polymer in the cell wall, but lignin and hemicellulose and the interactions between the cell wall polymers are indispensable for the overall performance of the cell wall. Hence one of the future challenges in this field is to understand the interactions of the polymers in the cell walls. Apart from novel analytical techniques, understanding the mechanism of cell wall polymer biosynthesis can also contribute to our understanding of the molecular interactions in the cell wall.

1.4 Cellulose biosynthesis

Cellulose biosynthesis is found in both prokaryotes and eukaryotes. In all cellulose synthesizing organisms, ß-1,4-glucan chains arise from a cellulose synthase (CESA) complex (CSC) which is embedded in the plasma membrane (Gardner and Blackwell, 1974, Mueller and Brown Jr, 1980, Arioli *et al.*, 1998). Cellulose synthase proteins (CESAs) catalyse the synthesis of glucan chains using UDP-glucose as substrate, and multiple glucan chains arise simultaneously from a single CSC (Arioli *et al.*, 1998, Somerville, 2006, Allen *et al.*, 2020). In vascular plants, it was demonstrated by TEM images of freeze- fractured plasma membranes, that each CSC is composed of six globular rosette subunits forming a rosette approx. 25-30 nm in size, initially proposed to contain six CESAs per subunit (Mueller and Brown Jr, 1980). Assuming that all CESAs are active simultaneously in a CSC, then the number of CESAs consequentially should reflect the number of glucan chains in the so called elementary cellulose fibrils. Hence, it was estimated that each globular subunit may synthesize six glucan-chains assembling into an elementary cellulose microfibril of 36- glucan chains (Herth, 1983). However, recent studies have challenged the 36-CESA model based on the predicted area of a 36-chain microfibril cross section, which is too large to agree with recent experimental data. Several investigations have concluded that the elementary microfibril cross-sectional area of approx. 3 nm is too small to comprise 36 glucan chains. Instead, studies using NMR, FTIR, WAXS, small-angle neutron scattering (SANS) as well as computer modelling, have supported a 18 or 24 glucan chain model (Fernandes *et al.*, 2011, Newman *et al.*, 2013, Thomas *et al.*, 2013). The elementary CMFs emerging from a CSC cluster together into macrofibrils of varying thickness presumably influenced by the overall cell wall composition (Donaldson, 2007, Lyczakowski *et al.*, 2019).

In Arabidopsis, CESA1, -3 and -6 related are required for PCW synthesis, whereas CESA4, -7 and -8 are essential for SCW synthesis (Taylor *et al.*, 1999, Taylor *et al.*, 2003, Desprez *et al.*, 2007, Persson *et al.*, 2007). It was shown that three CESAs, forming a functional CSC subunit, are required to synthesize proper elementary CMFs, yet the exact arrangement and stoichiometry has long been unclear. The knowledge of the stoichiometry of the catalytic subunits is relevant in regard to unravel the exact assembly of the CSC and to draw conclusions about the formation of crystalline

microfibrils. By using quantitative proteomics such as co-immunoprecipitation and mass spectrometry, it was shown that in the PCW of Arabidopsis seedlings, the three respective CESAs proteins in a CSC subunit occur in equimolar amounts (Gonneau *et al.*, 2014). The same equimolar ratio was confirmed in SCW CESAs in Arabidopsis by performing quantitative immunoblotting (Hill *et al.*, 2014), suggesting a 1:1:1 stoichiometry CSC model in Arabidopsis. Recently, Zhang et al. (2018) quantified the CESA stoichiometry in *Arabidopsis*, aspen (*Populus tremula*) as well as in Norway spruce (*Picea abies*) applying quantitative peptide mass spectrometry. The 1:1:1 stoichiometry of CESA4, CESA7 and CESA8 in SCW of Arabidopsis stems and Norway spruce was validated. However, the stoichiometry of CESA4, CESA7a/b and CESA8a/b in differentiating xylem of aspen wood showed a 2:1:3 ratio instead, and additionally a 3:1:8 ratio in TW of aspen (Zhang *et al.*, 2018). These results suggest that CSC stoichiometry can be both species and tissue type specific. Interestingly, *in vitro* studies revealed that the heterologous expressed and purified *Populus trichocarpa* SCW CesA8 (PttCesA8) alone is able to synthesize glucan chains and bundle them into higher order CMFs. Using electron microscopy, several micrometres long CMFs emerging from globular particles could be visualized. These globular particles are assumed to consists entirely of several CesA8 proteins that form a homomeric complex (Purushotham *et al.*, 2016). Hence, it seems that a heteromeric CSC structure is not required for CESA activity and cellulose biosynthesis.

In addition to the CSC structure, the CMF properties may be affected by the cellulose biosynthesis process. The trafficking of CSCs to and from the plasma membrane can affect events including lifetime of CSCs, the glucan chain lengths (degree of polymerization, DP), cellulose crystallinity, potential interaction with other polymers and finally the total amount of cellulose (Lei *et al.*, 2015). Especially the length of the glucan chains is assumed to be determined by the lifetime and velocity of CSCs at the plasma membrane (Gu *et al.*, 2010, Bashline *et al.*, 2014). The CSC lifetime during PCW biosynthesis was reported to be >15 min and potentially up to 40 min (Paredez *et al.*, 2006, Bashline *et al.*, 2014). Cellulose biosynthesis and alignment of the emerging CMFs into the cell wall also relies on additional structural proteins, which are discussed in the next section.

1.4.1 Proteins involved in cellulose biosynthesis

In addition to the catalytic CESA units, the whole cellulose synthase machinery relies on additional CSC related proteins, which facilitate and organize cellulose synthesis, CMF deposition and arrangement. These components have been identified through analysis of mutants, which exhibit reduced cellulose content and/or CSC velocity in the plasma membrane, alterations in CMF orientation in the cell wall and defects in cell expansion (for review see Allen *et al.* (2020)). As a result, the current models(s) of cellulose biosynthesis contain several components of which KORRIGAN (KOR), COBRA (COB), CELLULOSE SYNTHASE MICROTUBULE UNCOUPLING (CMU) and CELLULOSE SYNTHASE INTERACTING PROTEIN 1/POM-POM2 (CSI1) are the most studied (**Figure 6**).

The plasma membrane bound endo-1,4-ß-D-glucanase KORRIGAN (KOR) was shown to have an important role in cellulose biosynthesis (Nicol *et al.*, 1998, Takahashi *et al.*, 2009). Arabidopsis *kor-1* mutants exhibited reduced cellulose content, CESA velocity in the plasma membrane and growth (Nicol *et al.*, 1998, Paredez *et al.*, 2008, Vain *et al.*, 2014). A fluorescent labelling study visualized the interaction of KOR with the PCW CESAs during cellulose biosynthesis and suggested an involvement in CSC trafficking (Vain *et al.*, 2014). COBRA (COB) is an apoplastic glycosylphosphatidylinositol (GPI)-anchored protein. In Arabidopsis *cob* mutations cause cell expansion defects and reduced crystalline cellulose content (Benfey *et al.*, 1993, Roudier *et al.*, 2002). COB was suggested to be involved in the orientation of CMFs as well as formation of crystalline cellulose (Roudier *et al.*, 2005, Ben-Tov *et al.*, 2018). However, the exact underlying mechanism that determines cellulose crystallinity is not yet understood. CELLULOSE SYNTHASE MICROTUBULE UNCOUPLING (CMU) proteins are considered to be required for proper cortical microtubule (cMT) spacing during cellulose biosynthesis in order to withstand the forces generated by the movement of the CSC (Liu *et al.*, 2016).

Evidence mainly from Arabidopsis has established an important role for cortical microtubules (cMTs) during cellulose biosynthesis. cMTs are a specific feature of plant cells that are attached to the plasma membrane enchasing the cell (Shaw *et al.*, 2003). Already in the 1960s, it was observed that cMTs and CMFs were aligned. This correlation of the organization of

cMTs and CMFs led to the hypothesis that the organization of nascent CMFs might be guided by cMTs (Green, 1962, Ledbetter and Porter, 1963). By using immunofluorescence microscopy, CESAs and cMTs could be visualized simultaneously indicating that CSCs move through the plasma membrane following tracks parallel to cMTs. These studies supported the assumption of a mechanism that guides the emerging CMFs alongside the cMTs (Funada *et al.*, 1997, Barnett and Bonham, 2004, Paredez *et al.*, 2006). One of the cellulose biosynthesis associated proteins that was studies in this thesis is the CELLULOSE SYNTHASE INTERACTING PROTEIN 1/POM-POM2 (CSI1) that interacts with cMTs. *CSI1* was identified to be co-expressed with primary *CESA* genes. CSI1 acts as a linkage between cMTs and CSCs and facilitates the alignment of CESAs and cMTs during PCW synthesis as well as during the initial phase of SCW patterning in protoxylem vessels in Arabidopsis (Gu *et al.*, 2010, Li *et al.*, 2012). Arabidopsis *csi1* mutants show a delocalization of the CSCs from the cMTs affecting cell wall patterning in the PCW resulting in impaired cell expansion and twisting hypocotyls and rosette leaves (Bringmann *et al.*, 2012b). However, even in the absence of cMTs, CSC trajectories were observed to be maintained, indicating an additional microtubule-independent guidance of the CSC (Schneider *et al.*, 2017).

Chan and Coen (2020) recently provided evidence for a dual mechanism that guides cellulose biosynthesis in the plant cell wall. In the absence of cMTs, an autonomous mechanism takes over the guidance of the emerging CMFs into the cell wall where they most likely follow along the most recently synthesized CMFs. The guidance by cMTs seems to be dominant over the autonomous mechanism (Chan and Coen, 2020). These findings are supported by a previous study, which showed that once the cell wall patterns of the SCW are established, they can be maintained even in the absence of CSI1. That means that CSI1 is not required for the formation of SCW deposits, but most probably during the transition between primary to SCW (Schneider et al. 2017). In addition to its guiding function, Gu et al. (2010) could show that in Arabidopsis *csi1* mutants, the average speed of the CSC in the plasma membrane was reduced (Gu et al. 2010).

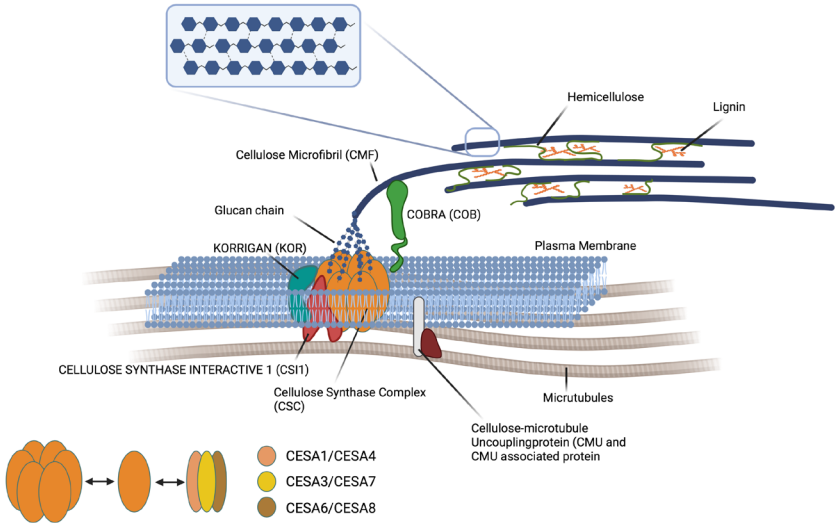

Figure 6: Model of cellulose biosynthesis and associated proteins.
Created with BioRender.com

1.5 Mechanical properties of wood

It is of both biological and industrial interest to understand the underlying mechanisms that affect the mechanical performance of wood. The mechanics of wood exhibit significant variation depending on the tree species, its age, developmental stage, genetics and environmental factors.

Two key parameters used to characterise mechanical wood properties are modulus of elasticity (MOE) also called Young´s modulus and tensile strength (ultimate stress) (Cave, 1969, Köhler and Spatz, 2002). These parameters can be extracted and calculated from a so-called stress-strain curve which can be obtained when applying force to a material to measure its deformation. Stress is a measure of the force applied per area of the material whereas the strain describes the material deformation over time with respect to its original length. The modulus of elasticity (MOE) is a measure of stiffness, which in turn is defined as the resistance of a material to deformation. The MOE is calculated from the slope of a stress-strain curve.

Normally, a stiffer material will have a higher MOE. The tensile strength of a material is the ultimate stress at the point before failure in a stress-strain curve (Burgert, 2006). Mechanical performance of wood can be measured in the longitudinal direction, that means parallel to the fibre length, and in radial direction perpendicular to the longitudinal axis of the fibres. Wood mechanical properties are often determined by applying force or pressure to either thin wood sections or whole wood boards or blanks. These measurements can be performed with so called tensile test stages or 3- point bending devices (**Figure 7**).

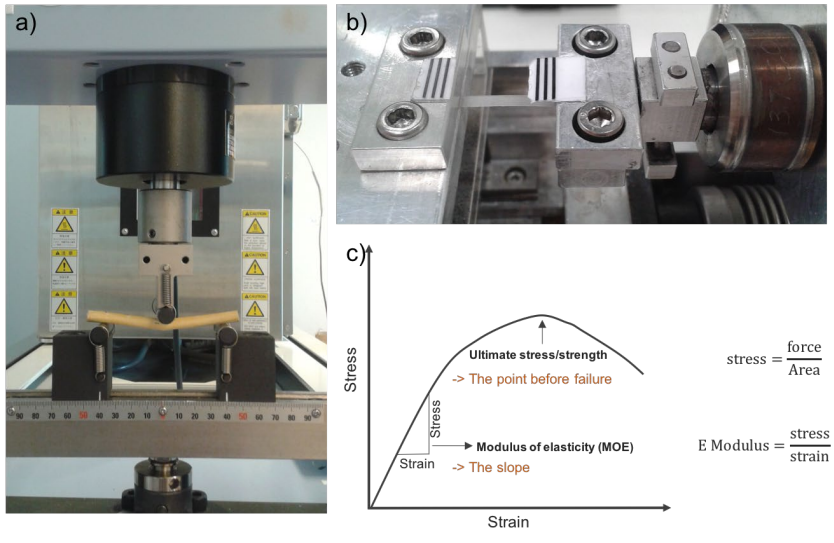

Figure 7: Mechanical testing of wood samples.
a) Three-point bending testing device with young wood stem. b) Micro-tensile testing stage for thin longitudinal wood section. c) Stress-strain curve and mechanical properties.

The hierarchical structure of wood needs to be dissected into the different levels to understand the mechanical performance of wood as a whole. The factors that can affect the wood mechanical properties are related to wood anatomy such as cell dimensions and wood density on the macro scale level, and also the composition and ultrastructural architecture of the polymers within the cell wall on the micro and nanoscale level. These factors determine both the individual xylem fibre micromechanics as well as the overall wood mechanical properties.

On the anatomical level, fibre dimensions and wood density play an important role for the mechanical performance of wood (Beery *et al.*, 1983). These factors are often defined by the developmental stage of the tree and seasonal changes. The proportions of juvenile and mature wood as well as earlywood and latewood affect significantly the mechanical properties. In young trees the xylem fibres are often shorter and cell walls thinner while as the tree ages the fibres become longer with thicker cell walls, leading to increased wood density (Jozsa and Middleton, 1994). In addition, early wood is comprised of wider xylem fibres with thinner cell walls compared to latewood xylem fibres. Thus, denser latewood rings are alternating with more porous earlywood rings, causing local mechanical variation across the wooden stem (Biblis, 1969, Jozsa and Middleton, 1994, Groom *et al.*, 2002). Another factor that plays an important role for the mechanical properties is the wood grain which is defined as the orientation of the fibre cells in relation to the longitudinal axis of the stem. Deviation of the fibre orientation from the direction of applied force is known to cause a decrease in strength and MOE (Pope *et al.*, 2005, Kretschmann, 2010, Mania *et al.*, 2020).

At the ultrastructural level, the thick SCW of xylem fibre cells and the properties and orientation of the CMFs have a significant impact on the mechanical strength of the wood. Thus, understanding the architecture and structure of the SCW is of importance for understanding wood mechanical properties. Its high abundance, unique chemical structure and arrangement in the wall (which is reviewed in more detail in section 1.4) makes cellulose the main load bearing polymer in the SCW. Within the SCW, the S2 layer accounts for more than 80% of the fibre wall weight, contributing most to the longitudinal mechanical properties of the wooden tissue (Fengel and Stoll, 1973, Sorieul *et al.*, 2016). Thus, the composition and ultrastructural

arrangement of the cell wall polymers in the S2 layer is of central importance for the overall mechanical performance of wood.

The cellulose MFA in the S2 layer is critical for the mechanical properties with respect to the longitudinal fibre axis (Lichtenegger *et al.*, 1999, Evans and Elic, 2001, Barnett and Bonham, 2004). A low average MFA (5-15°) in the S2 layer gives stiffness and strength but makes the wood more brittle. A high MFA (>15°) results in flexibility and toughness, which means that the wood is more resistant to fracture during deformation (Cave, 1968, Lichtenegger *et al.*, 1999, Burgert, 2006). The cellulose MFA in the S2 layer clearly dominates the longitudinal mechanical properties of the wood (Cave, 1968, Cave, 1969, Preston, 1974, Bergander and Salmén, 2002, Xu *et al.*, 2011). It was shown that the longitudinal stiffness of softwood cell walls increases approx. fivefold as the cellulose MFA decreases from 40° to 10° (Cave, 1968, Cave, 1969). However, several investigators have observed that the thinner S1 and S3 layer with their much more horizontally oriented CMFs are important to strengthen the cell laterally against deformation and mechanical stress (Booker and Sell, 1998, Bergander and Salmén, 2002). Hence, the S1 and S3 layers are not neglectable for the overall mechanical properties of wood.

In addition to the effect of cellulose MFA in a single fibre cell, there are also MFA variations between juvenile and mature wood that affect the mechanical properties. A significant difference in the Young´s modulus was observed in longitudinal wood sections that were taken from different distances from the pith outwards (Cave and Walker, 1994). Cave and Walker (1994) argued that this is due to the variation in the MFA of the S2 layer. Indeed, in both angiosperm and gymnosperm trees, the MFA was measured to be larger in juvenile wood which is the innermost part of the stem and formed when tree was young and thin and needed to be flexible to withstand winds and mechanical stresses without breaking. As the tree stem grows in diameter and forms mature wood, the cellulose MFA in the xylem fibres decreases providing stiffness (Lichtenegger *et al.*, 1999, Evans *et al.*, 2000, Bonham and Barnett, 2001). Thus, the Young's modulus increases. In addition, by using XRD, the MFA in the SCW of both angiosperms (*Eucalyptus* sp.) and gymnosperms (*Picea abies*) were measured to be significantly lower in latewood compared to earlywood (Stuart and Evans,

1994, Lichtenegger *et al.*, 1999). These observations indicate to a biological mechanism that allows the tree to mechanically adapt to the developmental stage and environmental circumstances. It can be concluded that the MFA and wood density between individual fibre cells, alternating growth rings (early wood and late wood) as well as developmental stage (juvenile wood and mature wood) all affect wood mechanics and lead to a large within-tree variation.

In addition, it is assumed that the cellulose DP and cellulose crystallinity have an impact on the mechanical properties of wood. The DP reflects the length of the individual glucan chain while cellulose crystallinity is defined as the ratio of crystalline to crystalline plus amorphous content by volume (Rongpipi *et al.*, 2019). Increased cellulose DP and crystallinity were reported to positively affect the mechanical properties of cellulose fibres and cellulose derived materials (Andersson *et al.*, 2003, Henriksson *et al.*, 2008). Another study showed a correlation between cotton fibre strength and the DP of the isolated cellulose (Benedict *et al.*, 1994). Thus, it could be assumed that crystallinity and DP also affect the mechanical properties of the native wood. However, the exact contribution of these parameters to the mechanical properties of wood are not fully understood.

While cellulose is known to be the main load bearing polymer in the cell wall, the role of lignin and hemicellulose and their impact on wood mechanics is less well understood. Due to the complex and intricate relationship between the matrix polymers in the cell wall, it is difficult to study their impact on the mechanical properties separately *in situ*. Besides, isolated cell wall polymers might not have identical features as *in situ*. This makes it problematic to relate their isolated mechanical properties in order to relate to the overall wood mechanical properties (Cousins, 1976). These challenges in mind, lignin is considered to give stiffness and strength to the cell wall, yet it is mechanically weaker than cellulose (Gibson, 2012). Wood from genetically modified poplar trees with 30% less lignin exhibited reduced tensile stiffness (Bjurhager *et al.*, 2010). However, the reduction in lignin also implied a significant change in wood density which was correlated with the mechanical weakness. Horvath et al. (2010) observed that a reduction in lignin content from 21% to 15-17% leads to a significant reduction in the mechanical wood properties in aspen trees whereas

structural modifications, implying an increase in the S-/G- lignin ratio, only had minor effect on the mechanical properties of the wood (Horvath *et al.*, 2010). Another study of genetically modified poplar trees, exhibiting structural changes in the lignin network that was assumed to affect the crosslinking with hemicellulose, found a reduction in the mechanical properties whereas density and cellulose MFA were not affected. These results indicate that the structural changes of lignin might directly affect the mechanical properties of wood (Özparpucu *et al.*, 2017). Additionally, microtensile tests coupled to Raman spectroscopy can be used to investigate the impact of mechanical deformation on the cell wall matrix polymers on the molecular level by changes in peak intensity. This technique revealed that lignin, in contrast to cellulose, does not seem to have a load bearing function in the cell wall (Burgert, 2006).

The role of hemicellulose on the mechanical performance of wood began to be elucidated only recently. Hydrogen bonds between cellulose and hemicellulose and electrostatic bonds between lignin and hemicellulose are known to affect the mechanical properties of cell walls (Simmons *et al.*, 2016, Kang *et al.*, 2019). Alterations of the cell wall hemicellulose composition may affect the structure and links between cellulose, hemicellulose and lignin, cellulose MFA as well as cell wall density and thus affect the mechanics of the cell wall and finally of the wood. A recent study by Berglund et al. (2020) investigated the contribution of hemicellulose on the mechanical properties of the SCW. By using purified hemicelluloses (xylans and glucomannans) from both hard and softwoods, that were incorporated into self-assembled bacterial cellulose networks, they attempted to mimic the chemical structure of the plant SCW before lignification and thus study the contribution of hemicellulose on the mechanical properties. The results showed that mannans increase the MOE under compression while xylans increase the ductility, meaning the ability to deform under tension. The explanation for these differential effects of xylan and mannans on the cellulose network may reside in the ultrastructure of the hemicellulose network and interactions with CMFs. The specific structural features of xylans and mannans create both rigid and more flexible domains within the cellulose network which determine the micromechanical behaviour under tension and compression. Under compression, it is proposed that xylan prevents adhesion and thus separates individual CMFs while

mannans increase adhesion by “gluing” CMFs to each other (Berglund *et al.*, 2020).

Taken together, the unique chemical structure of cellulose, the MFA in the SCW and wood density are the most important contributors to the mechanical performance of wood. It is also clear that lignin and hemicellulose contribute to the overall mechanical properties, but further work is needed to dissect the mechanism of their contribution. Eventually, it is the interplay of the cell wall composition, ultrastructural architecture and linkages between the matrix polymers as well as cell dimensions, wood density and the overall hierarchical wooden structure that defines the mechanical properties of wood.

1.6 Structure of cellulose

In 1838, the French chemist Anselme Payen (Payen 1838) was the first to describe the composition and chemistry of cellulose as a ß-1,4-linked glucose polymer. Later it was shown that cellulose is a liner polysaccharide built up of hundreds to thousands glucose molecules (Staudinger, 1934, Gardner and Blackwell, 1974). Cellulose has properties that differ from other organic polymers giving it unique biological and commercial value. What makes cellulose such an outstanding raw material is largely owning to its unique chemical structure. Regardless of the species, the primary cellulose structure is always the same: a linear unbranched polymer of glucose units connected via ß-1,4- glycosidic bonds whereas cellobiose, a glucose dimer, is often referred as the repeating unit of the cellulose (Gardner and Blackwell, 1974). Each glucose unit is rotated 180° relative to the previous unit forming a flat, so called two-fold screw ribbon (Hermans *et al.*, 1943, Allen *et al.*, 2020). The total number of repeating glucose units in a single cellulose polymer is defined as the DP and consequently reflects the length of the cellulose molecule. The cellulose DP varies significantly depending on the origin of the raw material as well as on the extraction method. In primary plant cell walls, the DP is thought to range from several hundred up to 8000 glucose units, whereas in secondary plant cell walls the DP can be at least up to 14,000-15,000 (Sjöström, 1993, Brett, 2000, Somerville, 2006). A DP of 2000 corresponds to a length of approx. 1 mm.

The primary structure gives rise to the secondary structure which gives cellulose its extraordinary strength. Intra- and inter molecular hydrogen bonds stabilize the glycosidic bonds and make the cellulose chains stiff and rigid and partially crystalline (Atalla and VanderHart, 1984). These properties make cellulose insoluble in water and in most organic solvents. The individual glucan chains, arising from the CSC, assemble into elementary CMFs that were shown to be approx. 3 nm in width in vascular plants (Donaldson, 2007, Fernandes *et al.*, 2011, Thomas *et al.*, 2013). These elementary CMFs aggregate into higher order structures as microfibrils and macrofibrils that were measured to be approx. 10-20 nm and up to 60 nm in width (Fahlén and Salmén, 2005, Donaldson, 2007, Fernandes *et al.*, 2011).

In 1858 Carl von Nägeli described the crystalline structure of cellulose based on polarized light microscopy (von Nägeli *et al.*, 1858). This was later confirmed by X-ray crystallography (Nägeli, 1858, Meyer and Misch, 1937, Wilkie, 1961). Cellulose crystallinity was measured to range from 36-51 % in poplar trees by using XRD (Jin and Pascal Kamdem, 2009). It is assumed that CMFs contain both highly ordered (crystalline) regions and less ordered (semicrystalline or amorphous) regions, but the native arrangement of both forms is still debated. Two models describing the possible arrangement of crystalline and amorphous regions are currently suggested. One assumption is that paracrystalline cellulose is located on the surface of the cellulose fibrils surrounding a highly crystalline core (Park *et al.*, 2010, Fernandes *et al.*, 2011). The second one is based on amorphous segments interrupting the highly ordered crystalline parts (Atalla and VanderHart, 1984, Larsson *et al.*, 1997, Wickholm *et al.*, 1998, Park *et al.*, 2010, Fernandes *et al.*, 2011). In favor of the second model, cellulose disintegrates into very short crystalline structures after partial acid hydrolysis (Bondeson *et al.*, 2006, Moon *et al.*, 2011). Still, both arrangements could be occurring simultaneously. It is not known yet how the crystalline and amorphous regions are arranged within the fibrils. It is further unclear how the glucan chains form such a highly organized and crystalline structure that dominates in plant cell walls. It might be a spontaneous or an organized process controlled by proteins and/or other cell wall matrix components.

In the G-layer of TW the cellulose crystals in elementary CMFs were measured to be significant larger (6.5 nm) than in S2 layer (3.1 nm). These results suggested that the glucan chain number in the CMFs are increased by fourfold in the G-layer (Müller *et al.*, 2006, Clair *et al.*, 2010). Müller *et al.* (2006) assumed that this might be due to an association of four CSCs during G-layer formation. It was further assumed that the strikingly higher degree of cellulose crystallinity in the G-layer might be either due to a fundamental difference in cellulose biosynthesis or due to the very low amount of hemicellulose and virtual absence of lignin (Joseleau *et al.*, 2004). In the S-layers of NW CMFs are separated by hemicelluloses and perhaps not interacting laterally with each other to the same extent. In TW G-layer the reduced number of interacting matrix polymers may promote a more tight interaction of CMFs and lead to thicker and more crystalline CMF structure (Müller *et al.*, 2006).

In can be concluded that the precise ultrastructure of the CMFs is not yet fully resolved even though the structure of cellulose was described more than 150 years ago. Advances in techniques and methods, such as Fourier transform infrared (FTIR), ssNMR, infrared (IR), Raman spectroscopy, XRD (WAXS/SAXS), atomic force microscopy (AFM) and transmission electron microscopy (TEM) are providing an increasingly better understanding of cellulose structure, dimensions and interactions with the other cell wall matrix polymers (Nishiyama *et al.*, 2002, Ding and Himmel, 2006, Fernandes *et al.*, 2011, Newman *et al.*, 2013, Lyczakowski *et al.*, 2019). Nevertheless, the complex cell wall architecture and technical limitations of the current measurement methods still leave some open questions. For example, the native cellulose DP in the cell wall and how we can reliably measure it as well as the CMF orientation within the SCW layers still need to be answered in the future.

The unique mechanical and chemical characteristics of the CMFs, such as high crystallinity, exceptional stability, insolubility in water, low weight and density as well as ideal thermal properties has led to increased attention on CMFs as a sustainable, biocompatible and environmentally friendly raw material in modern nanotechnology. This topic will be discussed more thoroughly in the following chapter.

1.7 Nanocellulose

The concept of nanotechnology was introduced in 1959 by the American physicist and Nobel prize winner Richard Feynman who first introduced the idea of manipulating and controlling particles in the nano-scale range in his talk titled "There´s Plenty of Room at the Bottom" at the American Physical Society meeting (Feynman, 1961). The concept of nanotechnology also led to the study of nanomaterials, which are materials or substances made of units in the range below 100 nm. This small unit size and thus increased surface area per volume is used to create nanomaterials which show properties different from the original material the nanomaterial was derived from (Roduner, 2006). Many nanomaterials exhibit enhanced qualities such as high strength, low weight, increased surface area and increased chemical reactivity with other materials. These attributes have promoted increased activity in this research field evident in the exponential increase of publications and patents (Kostoff *et al.*, 2007, Youtie *et al.*, 2008, Zhu *et al.*, 2017, Klemm *et al.*, 2018). Fossil fuel-based nanomaterials are neither biodegradable nor sustainable and moreover potentially harmful to the environment and living organisms. This has promoted the interest in renewable, environmentally friendly and non-harmful sources for nano-scaled material. Especially, the demand for alternative materials in short-lived and disposable applications is expected to be important in order to replace conventional plastics.

Recently, highly crystalline nanocellulose, also termed cellulose nanocrystals (CNCs) and cellulose nanofibrils (CNF), has gained attention as an innovative and sustainable raw material for nanotechnology. CNCs and CNFs can be extracted from a vast variety of cellulose sources such as wood, crops, algae, or cellulose synthesizing bacteria (Klemm *et al.*, 2009, Chen *et al.*, 2016, Jozala *et al.*, 2016). Applications for nanocellulose based materials are steadily increasing, especially in packaging, hygiene products, absorbents or food additives as well as high-end products such as biomedical devices, filter systems, catalytic membranes, electronic devices and composites (Jonoobi *et al.*, 2012, Jonoobi *et al.*, 2015, Dufresne, 2017a, Voisin *et al.*, 2017, Klemm *et al.*, 2018).

1.7.1 Nanocellulose terminology and isolation

Nanocellulose is defined as defibrillated cellulose material or fragments in the nano-sized range of <100nm (Dufresne, 2017b). Nanocellulosic structures can differ widely in terms of dimensions, architecture and chemistry. In this thesis, I will refer to the international standards and concepts for nanocellulose, defined and determined in a roadmap developed and published by the Technical Association of the Pulp and Paper Industry (TAPPI) in 2011. The terms I use from TAPPI are cellulose microfibrils (CMFs), cellulose nanofibrils (CNFs) and cellulose nanocrystals (CNCs) (Boluk *et al.*, 2011).

It is important to distinguish between the terms CMFs (also called elementary cellulose microfibrils) and CNFs and CNCs. CMFs refer to the cellulose structure found *in vivo* in cellulose synthesizing organisms. The diameter of native elementary CMFs depends on the number of glucan chains, which is most likely restricted by the number of active CESAs in a CSC. In contrast to CMFs, the CNFs are isolated from cellulosic raw material and artificially processed. Thus, CNFs and CNCs are technological terms to describe the engineered structure or material purified from organic sources by either mechanical, chemical or enzymatic treatment respectively.

The CNC and CNF properties depend on the method used to extract them from the raw material. There are several methods established for the isolation of CNCs and CNFs. CMFs are assumed to contain highly crystalline and amorphous regions. CNCs are short and stiff, needle-like almost pure crystalline particles typically obtained by acid hydrolysis. As described in section 1.4, it is thought that acid hydrolysis causes the cleavage of the glycosidic bonds in the amorphous parts of CMFs while leaving the crystalline regions intact (Bondeson *et al.*, 2006, Moon *et al.*, 2011). CNCs can be very different in their dimensions and properties depending on their source and preparation method. The width of CNC can range from 3 - 70 nm, while the average length varies from only tens of nanometres to several micrometres (Habibi *et al.*, 2010, Isogai, 2021).

CNFs are often manufactured using a chemical pretreatment of the cellulosic raw material, such as wood pulp, followed by a mechanical disintegration of the individual CMFs. These treatments are thought to preserve the non-

crystalline parts in the microfibrils. Isolated CNFs can be as thin as 1-2 nm but also up to tens of nm in width and typically >500 nm in length (Jonasson *et al.*, 2020, Isogai, 2021).

A partial removal of non-cellulosic compounds is required to purify CNFs. Treatments using NaOH, several filtrations and washing steps to remove other cell wall matrix polysaccharides are commonly followed by a bleaching step using $NaClO_2$ to wash away lignin and proteins. In order to obtain thin CNFs, the purified cellulose suspension requires an additional mechanical treatment. Mechanical disintegration, also called nanofibrillation, can be achieved by using a high-pressure homogenizer. This procedure is based on pushing the cellulose suspension repeatedly, under high pressure, through a narrow valve which leads to fibrillation of the cellulose network (Dufresne, 2017b, Dufresne, 2019).

Altering the native cellulose structure while isolating nanocellulose is a common issue affecting the native crystalline cellulose structure as well as causing a decrease in the cellulose DP (Kumar *et al.*, 2009, Hubbell and Ragauskas, 2010, Klemm *et al.*, 2018) . These changes are often caused by the harsh chemical pre-treatment required to separate the CMFs from the cell wall matrix. Another drawback hindering commercialisation at large scale is the need of environmentally harmful chemicals and energy consuming mechanical treatments during the extraction process. This is one of the main research incentives to develop and improve more sustainable and efficient extraction methods (Phanthong *et al.*, 2018).

A method to isolate CNFs that gained attention in the last decade is the chemical pretreatment with 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) prior to the mechanical disintegration of the cellulose fibrils. TEMPO mediates selective carboxylation of the C6- hydroxyl groups of cellulose and thus facilitates the separation of the individual cellulose fibrils during the mechanical disintegration (Saito *et al.*, 2006, Saito *et al.*, 2007, Isogai *et al.*, 2011). The ease of fibrillation of the nanocellulose depends on the carboxyl content introduced by TEMPO. Hence, an efficient TEMPO pretreatment allows a more energy-saving mechanical treatment compared to other methods (Saito *et al.*, 2007, Isogai *et al.*, 2011, Isogai, 2013, Isogai, 2021). Compared to other pretreatment methods, such as ammonium persulfate

oxidation, it was additionally shown that TEMPO-mediated oxidation improves the DP preservation of the CNFs, which is thought to be of advantage for the mechanical performance of the final product (Henriksson *et al.*, 2008, Isogai *et al.*, 2009, Filipova *et al.*, 2020). In addition, it has been shown that TEMPO also simultaneously delignifies the wooden raw material (Ma *et al.*, 2012, Jonasson *et al.*, 2020). This feature makes an extra delignification step redundant.

1.7.2 Nanocellulose properties

Nanocellulose has the potential to reinforce or even replace established materials such as Kevlar®, carbon nanotubes and common plastics (Moon *et al.*, 2011). The excellent stability and strength of nanocellulose material is mainly owed to the crystalline cellulose structure (Trache *et al.*, 2017). The MOE of native cellulose crystals was reported to range from 56-220 GPa with an average of approx. 130 GPa and tensile strength of approx. 10 GPa (Dufresne, 2017a). Additionally, the enormous reinforcing capacity of nanocellulose derives from the low weight and density and a very large surface area (volume: surface ratio) (Dufresne, 2013). In addition, pure cellulose has a hydrophilic surface making it a good water absorbed (Biermann *et al.*, 2001). The tensile strength of CNCs was reported to range from 7.5-7.7 GPa which is almost double as high as for Kevlar® and steel wires (Moon *et al.*, 2011). CNFs were measured to have a MOE of 100 GPa (Dufresne, 2017a). The strength-to-weight ratio of cellulose nanomaterials was measured to be even eight times higher than stainless steel (Dufresne, 2019).

1.8 Wood as a raw material for CNF production

Wood fibres could potentially be used as an abundant raw material for nanocellulose isolation. The advantage of wood is the renewability and the well-established processing infrastructure. Additionally, the harvest and processing of trees is not restricted to seasons and compared to many crop plants, trees are very adaptable to environmental changes. However, the structure and chemistry of wood represents some formidable challenges for CNFs extraction. The procedure for extracting nanocellulose from wood typically starts with delignified wood pulp, which is mechanically disintegrated to obtain CNFs. In order to make cellulose more accessible and thus reducing energy consumption during mechanical disintegration, a chemical pre-treatment is necessary. One of the major limitations of wood in this respect is its recalcitrance. Structural factors such as call wall matrix polymer interactions, cellulose crystallinity and DP as well as chemical factors such as cell wall composition affect wood recalcitrance.

Lignin in particular may hinder nanocellulose production from wood. The tight association and complex structure of the cell wall matrix polymers and the hierarchical structure of wood require delignification, and deconstruction of the cell wall to release the CMFs. In multiple steps, the non-cellulosic part is separated and removed from the cellulosic part, typically using highly energy consuming, environmentally harmful and cost-intensive extraction methods such as harsh alkali and acid treatments (Filson and Dawson-Andoh, 2009, Jiang *et al.*, 2010, Yu *et al.*, 2013). Especially the effective removal of lignin and disintegration of the CMFs from the wooden cell wall has been considered a major bottleneck in order to obtain high quality nanocellulose. Reducing wood recalcitrance to facilitate the extraction procedure by genetically modifying both lignin content and structure may be one way to improve the usability of wood for nanocellulose production. It was shown that genetically modified poplar trees with downregulated enzymes involved in the lignin biosynthesis pathway causing altered lignin structure, decreased the extraction chemicals use by 6% in the pulping process. The introduced structural changes were assumed to improve the solubility of lignin during the pulping (Pilate *et al.*, 2002). Other studies investigated the effect of a reduced lignin content as well as compositional changes caused by an increased S/G-lignin ratio in both poplar trees and Arabidopsis. These modifications showed to have a positive effect on

extractability during the pulping process (Huntley *et al.*, 2003, Mansfield *et al.*, 2012, Van Acker *et al.*, 2013). However, even if lignin modifications were shown to improve the extraction efficiency by reducing energy and chemical consumption, lignin alterations often result in impaired tree growth, reduced biomass, and ability to respond to biotic and abiotic stress factors (Hancock *et al.*, 2007, Coleman *et al.*, 2008, Moura *et al.*, 2010, Van Acker *et al.*, 2014).

The impact of hemicellulose on wood recalcitrance is less well understood and several studies came to contradictory conclusions. Depending on processing methods and raw material, it was reported that in some cases the removal of hemicellulose was more important than lignin removal while in other cases lignin removal improved cellulose purification (Wang *et al.*, 2012, Leu and Zhu, 2013, Li *et al.*, 2016a). However, other studies reported that hemicellulose is even beneficial for nanocellulose production. During nanocellulose extraction from both softwood and hardwood pulp, hemicellulose was reported to facilitate the mechanical fibrillation process in order to produce CNFs (Iwamoto *et al.*, 2008, Arola *et al.*, 2013). Hemicellulose is considered to space and thus hinder CMFs from aggregation (Kang *et al.*, 2019). Likely this structural feature eases the separation of CMFs during mechanical disintegration. Additionally, these results indicated that hemicellulose could enhance mechanical properties in CNF networks (Iwamoto *et al.*, 2008, Arola *et al.*, 2013, Dufresne, 2019). However, it is not well understood how hemicellulose exactly is located between the CMFs and how this affects the fibrillation process and final properties of CNFs. Additionally, the effect of different hemicelluloses on CNF isolation remains elusive.

Due to constant technical development of purification and fibrillation techniques nanocellulose extraction is predicted to become less chemistry intensive and energy consuming and thus more sustainable (Dhali *et al.*, 2021). The foundation of my PhD project was the vision that new tools for genetics-based modifications of the cell wall structure, such as targeted reduction and/or alteration of the lignin structure causing decreased wood recalcitrance may facilitate the extraction of nanocellulose. Targeted and rational breeding of trees as a raw material for nanocellulose production requires a detailed understanding of cell wall biosynthesis, ultrastructure and

interconnections of the cell wall polymers as well as their effect on the wood properties. At the start of this project little was known about the correlation between native cell wall structure and composition and the effect on extractability and quality of nanocellulose from the wood. It was envisioned that a multi-disciplinary approach of combining fundamental wood biology, including the study of underlaying genetical mechanisms defining cell wall structure and mechanical performance of wood, and material science, including developing more efficient extraction methods, is required to move towards sustainable use of wood for nanocellulose production.

2. Objectives

The aim of this thesis was to combine fundamental wood biology with material science in order to study and improve the effects of native wood properties and the underlaying genetic mechanisms influencing the extraction and performance of nanocellulose from wood. With this approach we also explored the possibilities of genetically improving nanocellulose production and the quality of the final product. I investigated the *Populus* CSI1 protein involved in cellulose biosynthesis and its effects on SCW formation and overall wood mechanical properties. In collaboration with material scientists at Luleå Technical University, I also investigated different *Populus* hybrids with varying wood properties as well as *CSI1RNAi* lines for nanocellulose production and nanocellulose performance.

Specific objectives:

Paper I: Investigating the function of the CELLULOSE SYNTHASE INTERACTIVE 1 (CSI1) protein on secondary cell wall formation in hybrid aspen and its contribution to wood mechanical properties.
Paper II: Investigating the potential suitability of aspen tension wood for nanocellulose production.
Paper III: Investigating a hybrid aspen population exhibiting different wood lignin contents on the extraction and performance of nanocellulose.
Paper IV: Investigating the suitability of hybrid aspen trees with decreased *PtCSI1* activity for nanocellulose production and how native wood cell wall properties reflect the performance and quality of cellulose nanofibrils.

3. Model Organisms

In this thesis, hybrid aspen (*Populus tremula x tremuloides*) (Paper I, IV), *Populus tremula* (Paper II, III), and *Arabidopsis thaliana* (Paper I) were used as model organisms. The most common model species in molecular plant science is the flowering plant *Arabidopsis thaliana.* The genome of Arabidopsis was completely sequenced in the year 2000. Its small genome size and well-established methods for genetically modification, such as Agrobacterium-mediated T-DNA insertion, RNAi gene silencing and CRISPR-Cas9 gene editing have made Arabidopsis the workhorse to study fundamental biological processes and pathways. Since many mechanisms and pathways are conserved throughout the whole plant kingdom, it is often possible to use Arabidopsis as a model organism to study universal processes. This opens possibilities for fundamental functional and comparative gene studies in order to validate findings from Arabidopsis in other species (Tuskan *et al.*, 2006, Jansson and Douglas, 2007). However, Arabidopsis has its limitations when it comes to the study of wood formation for which the perennial tree species *Populus sp*. is more suitable. Additionally, Populus is a commercially valuable tree in the northern hemisphere and the sequencing of the poplar genome in 2006 opened up new possibilities to study the underlying genetical mechanisms of SCW biosynthesis and wood formation (Tuskan *et al.*, 2006). Other advantages are its fast propagation time and growth compared to other tree species, and well-established gene editing tools, such as RNAi and CRISPR-Cas9 (Fan *et al.*, 2015).

In this thesis hybrid aspen (*Populus tremula x tremuloides*) *PtCSI1RNAi* mutants were used to study the function of CSI1 during SCW and thus wood formation (paper I) as well as their potential suitability for nanocellulose

production (paper IV). In order to confirm that *PtCSI1A* and *PtCSI1B* are functional orthologues of Arabidopsis *CSI1*, *Arabidopsis thaliana* was used to perform complementation experiments. Forest grown *Populus tremula* and a field grown hybrid aspen population (kindly provided by SweTree Technologies) respectively were used to investigate the role of native wood properties in terms of nanocellulose production (paper II, III).

4. Results and Discussion

4.1 CELLULOSE SYNTHASE INTERACTING I is required for wood mechanics and leaf morphology in aspen (paper I)

The aim of this study was to investigate the role of CELLULOSE SYNTHASE INTERACTING 1 (CSI1/POM2) in hybrid aspen (*Populus tremula x tremuloides*) during wood formation and cellulose biosynthesis (**paper I**). The possibility of reduced *CSI1* expression as a tool to improve nanocellulose isolation from wood was also explored in **paper IV**.

4.1.1 Populus CSI1 are functional orthologues of Arabidopsis CSI1

In Arabidopsis, CSI1 was shown to link cMTs and CSC and guide and align CMFs during PCW biosynthesis (Gu *et al.*, 2010, Bringmann *et al.*, 2012a, Li *et al.*, 2012). In hybrid aspen, PtCSI1A and PtCSI1B were identified as putative functional orthologues of the Arabidopsis CSI1 with 95% similarity at the amino acid sequence level (Fig. 1a), S1, paper I). In developing wood, based on publicly available transcriptome data, *PtCSI1A* and *PtCSI1B* show increasing expression level starting during cell expansion and peaking at the onset of SCW formation (Fig. 1b, paper I). Expression of *PttCSI1A* in the Arabidopsis *csi1* null mutant *pom2-4* complemented the Arabidopsis *pom2-4* phenotype which confirmed that PttCSI1A is a functional orthologue of the Arabidopsis CSI1 (Fig. S2, paper I). In order to study the role of *PttCSI1* in trees and during wood formation, transgenic hybrid aspen containing a 35s promoter driven *PttCSI1RNAi* construct targeting both *CSI1A* and *CSI1B*

were generated and grown under greenhouse conditions. Quantitative polymerase chain reaction (qPCR) analysis of *PttCSI1A* and *PttCSI1B* confirmed a significant reduction in the expression level in developing wood and leaves in three independent transgenic lines (Fig. 1c-f, paper I). Surprisingly, only a modest growth phenotype was observed for *CSI1RNAi* lines with slightly shorter stems compared to WT (Fig. 2a, paper I). However, a twisting leaf phenotype and defects in leaf epidermal pavement cell shape were more obvious and in line with earlier observations from Arabidopsis rosette leaves and hypocotyls pointing to a function during PCW biosynthesis, cell expansion and regulating pavement cell shape (Fig. 2b & c, paper I) (Fu *et al.*, 2005, Bringmann *et al.*, 2012b, Landrein *et al.*, 2013, Sampathkumar *et al.*, 2014). The absence of a major growth phenotype in *CSI1RNAi* lines may be explained by a recent study that provides evidence for a dual mechanism guiding cellulose biosynthesis. In the absence of cMT guided cellulose biosynthesis, an autonomous mechanism takes over the guidance of CMFs in the cell wall following the tracks of recently synthesized CMFs (Chan and Coen, 2020). This model is supported by a previous study showing that once the cell wall patterns of the SCW are established, they can be maintained even in the absence of CSI1 (Schneider *et al.*, 2017).

4.1.2 CSI1RNAi wood is mechanically weaker

In *CSI1RNAi* lines the expression level of Pt*CSI1A* and Pt*CSI1B* in the developing wood was measured to be 10%-30% of WT (Fig. 1c-f, paper I). Thin wood cross sections (20 µm) prepared from *CSI1RNAi* lines appeared to be more brittle perpendicular to the fibre direction during sample preparation compared to WT. In order to investigate the mechanical defect further, thin longitudinal wood sections (30 x 2 x 0.1 mm /L x W x H) were used for micromechanical tensile testing. These tests revealed a decrease in the mechanical wood properties in longitudinal direction apparent in a reduction of the MOE and ultimate stress/MOR. The MOE was reduced by approx. one-third (from 2.95 GPa in WT to 2 GPa in *CSI1RNAi* lines). MOR was significantly reduced in only one transgenic line, which also was in line with the lowest expression level of *PtCSI1A* and *PtCSI1B* in the wood (Fig. 4 a,b, paper I). However, no differences in overall wood anatomy and SCW structure (S1-, S2- and S3-layer) were observed based on light microscopy

and TEM images. Furthermore, no changes in the chemical cell wall composition were apparent between *CSI1RNAi* lines and WT which could explain the mechanical phenotype (Fig. 3., Table S2, paper 1).

4.1.3 CSI1RNAi wood density and average MFA are not changed

In order to understand the origin of the wood mechanical phenotype, wood density and cellulose MFA were measured as both are known to contribute to the mechanical performance of wood. Wood density was measured in the same samples as used for the mechanical testing and found not to be different in the *CSI1RNAi* lines compared to WT (Fig. 4c, paper I). Based on Arabidopsis hypocotyl studies, the cellulose MFA was hypothesized to be altered in *CSI1RNAi* lines. X-ray diffraction was used to measure the average MFA in the S2. However, no significant differences were found in both NW as well as TW between the transgenic lines and WT (Table 2, paper I). These results showed that the origin of the mechanical phenotype could not be ascribed to a change of the MFA in the SCW and overall wood density. Thus, the data does not support a role for CSI1 in CMF alignment during SCW formation in wood fibres in aspen trees. These observations agree with results from Arabidopsis showing that CSI1 is not needed to maintain the CMF pattern in xylem vessels (Schneider *et al.*, 2017). However, the question remains of how the MFA is established in the first place, and how the changes in MFA between the different cell wall layers are achieved. It is worth noting that X-ray diffraction is limited by its beam size. Thus, the MFA was measured as an average of the whole SCW whereas the S2 layer, due to its highest proportion, contributes most to the average MFA. A method with higher resolution may allow for MFA measurement in smaller steps throughout the SCW and separately for each S-layer. Especially the transitional layer between the S1-and S2-layer would be of interested as it was previously shown that CSI1 has a function during the initial phase of SCW patterning in Arabidopsis xylem vessels (Schneider *et al.*, 2017).

4.1.4 CSI1RNAi reduced cellulose degree of polymerization and longitudinal xylem fibre area

In Arabidopsis *csi1* hypocotyls, the speed of YFP labelled CESA6 subunits was measured to be reduced in the plasma membrane (Gu *et al.*, 2010, Lei *et al.*, 2013). Thus, if the CSC in *CSI1RNAi* lines is slowed down during wood formation, this could potentially cause shorter cellulose chains if lifetime of the CSC is not increased. To test this hypothesis, the cellulose DP in wood was determined by measuring the absolute molecular weight of the extracted cellulose using size exclusion chromatography (SEC) coupled to a laser light scattering (LS) detector. The obtained data indicated a shift of the molecular weight distribution towards slightly shorter CMFs in *CSI1RNAi* lines compared to WT. Interestingly, these results correlate with the reduction in the ultimate stress in line 1 and line 3 and support a role for cellulose DP in wood mechanics (Fig. 5, paper I). The reduction in cellulose DP could be ascribed, among other possibilities, to a reduced half-life of the CSC in the plasma membrane and/or reduced speed of the CSC and thus reduced rate of cellulose biosynthesis. However, it is likely that the chemical treatment with PAA during cellulose extraction and solubilization with DMAc decreases the native cellulose DP. Thus, it cannot be excluded that in CSI1RNAi lines, cellulose DP *in situ* is equal to WT but, due to potential structural defects of CMFs, more sensitive to the extraction method compared to WT.

Longitudinal wood fibre area was measured from macerated wood by using light microscopy images. A consistent reduction in fibre area by approx. 20% was shown in both *CSI1RNA*i lines (Table 2, paper I). These results suggest that the reduced expression of *CSI1* impaired cell expansion in fibre cells which is in line with earlier studies from Arabidopsis confirming a function during PCW biosynthesis for CSI1.

In this study we showed that in hybrid aspen a reduction of *PtCSI1* causes impaired mechanical wood properties evident as a decrease in both the elastic modulus and ultimate stress in thin wood sections. The mechanical phenotype could not be linked to changes in wood anatomy, cell wall composition, density or average cellulose MFA. We identified that *CSI1RNAi* influences the length of CMFs apparent as reduced cellulose DP. Since CMFs are the main load bearing components in the cell wall, we hypothesized that a reduction in cellulose DP contribute to the mechanical

weakness of the wood. It is also possible that structural changes in CMFs caused by *CSI1RNAi* affect interactions with the other matrix polymers and thus, together with a reduced DP, weakening of the wood. Additionally, we cannot exclude that the remaining expression of *CSI1* in the developing wood (10%-30%) in *CSI1RNAi* lines was sufficient to maintain part of the functionality during SCW formation.

4.2 The effect of wood properties on cellulose nanofibril production (paper II and III)

In a collaboration with material scientists from Luleå Technical University I investigated the effect of natural variation in wood cell wall composition on extractability and properties of nanocellulose. The aim was to understand the effect of cell wall composition and ultrastructure on CNF isolation on CNF network properties. In **paper II** we compared CNF properties from tension wood (TW) and normal wood (NW) of *Populus tremula* (aspen). We hypothesized that the chemical and structural features of the G-layer, including almost pure cellulose and low amounts of hemicellulose and lignin as well as increased cellulose crystallinity, crystalline CMF thickness and DP may result in improved CNF properties. This idea is supported for example by Henriksson *et al.* (2008) who reported that a high DP improves the mechanical properties of nanocellulose derived papers. Thus, the increased cellulose DP in the G-layer could improve the mechanical properties of isolated CNFs from TW.

In **paper III** we report the analysis CNFs extracted from field grown hybrid aspen trees which display large variation in wood lignin content. A low lignin content was hypothesized to improve wood recalcitrance and thus facilitate the accessibility of cellulose during CNF isolation (paper III). Wood cell wall composition from the natural population of field grown hybrid aspen trees was analyzed by performing pyrolysis-GC/MS and subsequent principal component analysis (Fig. S1, TableS1, paper III). Six samples with the largest variations in terms of carbohydrates and lignin content were selected in order to study the effect of cell wall composition on nanocellulose extraction. The samples were classified into low (Sample ID 1), medium

(Sample ID 2-5) and high lignin (Sample ID 6) content with variations in lignin content from approx. 17-30 weight % (Table 1, paper III).

In both **paper II** and **III**, wood powder was directly oxidized by 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) prior to mechanical disintegration of the cell wall and nanofibrillation of the cellulose. Fibrillation efficiency and CNF network characteristics as well as morphology and mechanical properties were analyzed. These results established that a combination of TEMPO-catalyzed oxidation and mechanical fibrillation (chemical-mechanical treatment) is required to obtain a high yield of highly fibrillated CNFs. In the following I will summarize and discuss these results in more detail.

4.2.1 TEMPO-oxidation of wood samples and fibrillation behavior

TEMPO-catalyzed oxidation was previously shown to simultaneously and efficiently carboxylate cellulose and delignify wood powder (Ma *et al.*, 2012, Jonasson *et al.*, 2020). We discovered that cellulose from TW powder is significantly more difficult to fibrillate compared to NW apparent as a lower yield of fine CNFs from TW (21 weight %) compared to CNFs obtained from NW (31 weight %) (Fig. 2, paper II). This means that in NW CNF suspensions a larger percentage of the total solids is comprised of thin CNFs compared to TW CNF suspension. Another indicator for a reduced fibrillation efficiency is the lower viscosity observed in TW CNF suspensions compared to the NW CNF suspension (Fig. 4, paper II). These results indicate that TW cellulose is less accessible to TEMPO oxidation compared to NW cellulose. This may be associated with the structural and chemical differences of the TW G-layer. In the S-layer of NW, individual CMFs are thought to be separated and spaced by hemicellulose in particular xylan (see section 1.3.3). Thus, the lower amount and/or altered structure and composition of hemicellulose in the G-layer could make the cell walls in TW more recalcitrant compared to NW. Additionally, increased cellulose DP and thicker crystalline CMFs measured by Foston *et al.* (2011) and Zhang *et al.* (2018) respectively may contribute to wood recalcitrance during the chemical-mechanical treatment.

TW contained significantly lower amounts of xylose (60.7 weight %) compared to NW (72.6 weight %) but higher amounts of galactose (2.4 weight %) than NW (0.6 weight %) (Table 1, paper II), which is in agreement with previous measurements (Gorshkova *et al.*, 2015). Gorshkova et al. (2015) showed that, xylan is indeed the most abundant hemicellulose in both TW and NW but absent from the G-layer. This most likely explains the overall lower amounts of xylan in TW compared to NW (Gorshkova *et al.*, 2015). Galactan was found to be highly abundant in the G-layer of TW but not in the S-layers of either TW or NW (Arend, 2008, Gorshkova *et al.*, 2015). The absence of xylan in the G-layer may increase the lateral interaction between individual CMFs which in turn could underlie the increased CMF crystalline diameter in TW. The increased crystalline CMF thickness may hinder TEMPO oxidation and mechanical fibrillation and thus make TW more recalcitrant compared to NW. This hypothesis is supported by previous studies which showed that hemicelluloses facilitate the mechanical fibrillation process during CNF production most likely by preventing aggregation of CMFs (Iwamoto *et al.*, 2008, Arola *et al.*, 2013, Kang *et al.*, 2019).

The increased lignin content in NW compared to TW did not seem to hinder the nanofibrillation process. This was surprising since a low lignin content was assumed to reduce wood recalcitrance and thus facilitate CNF isolation. However, we discovered that high lignin content correlated with improved nanofibrillation (paper III). Viscosity was higher in CNF suspension from wood with higher lignin content (Fig. 1 a, paper III). The same trend was shown for the nanofibril yield which slightly increased with increasing initial lignin content (Fig. 1 b, paper III). Thus, high lignin content appears to correlate with improved nanofibrillation. However, the process yield from initial dry wood decreased with higher initial lignin content, which may be explained by a corresponding lower amount of cellulose with increasing lignin content and the removal of lignin during TEMPO oxidation (Fig. 1b, paper III). For all samples, < 1 weight % lignin was detected after TEMPO oxidation illustrating an efficient delignification of the wood powder (Table 2, paper III). These results support that TEMPO- mediated oxidation, apart from cellulose carboxylation, can also be used to delignify wood. These results encourage further investigation of how wood lignin content influences CNF preparation and properties. However, at this point it is

important to note that an effect of improved CNF fibrillation was observed when comparing the most extreme lignin differences. More wood samples with varying lignin contents should be analyzed as it remains possible that other, yet to be identified, factors may also influence the processing.

4.2.2 CNF morphology

CNFs cross-sectional diameter was measured using AFM microscopy. The finest CNFs isolated from TW exhibited a smaller cross-sectional diameter compared to the finest CNFs from NW. Fig. 3 (paper II) shows the size distribution and average height (thickness) of CNFs isolated from TW and NW respectively. The average height of TW and NW CNFs were measured to be 1.2 nm and 1.6 nm respectively (Fig. 3, paper II). However, TW CNFs contained higher amounts of thicker fibrils ($\geq$ 10 nm) making the TW CNF suspensions more heterogenous in size (Fig. 4, paper II). The thicker CNF structures in TW samples most likely derive from insufficiently carboxylation of CMFs during TEMPO oxidation. This may be due to decreased TEMPO accessibility of TW CMFs, potentially due to increased crystallinity and DP.

Elementary CMFs in NW were measured to be on average 3 nm in diameter using X-ray diffraction (Donaldson, 2007, Fernandes *et al.*, 2011, Thomas *et al.*, 2013). In the G-layer of TW, CMFs were measured to be significantly larger (6.5 nm) than in the S-layer of NW suggesting structural differences of the CSC during cellulose biosynthesis and/or CMF assembly (Müller *et al.*, 2006, Clair *et al.*, 2010, Zhang *et al.*, 2018). In NW, elementary CMFs emerging from a CSC cluster together into macrofibrils varying in size depending on the overall cell wall composition. Both hemicellulose and lignin were shown to contribute to the size of cellulose macrofibrils (Donaldson, 2007, Lyczakowski *et al.*, 2019).

The CNF diameter from both low and high lignin CNFs was measured to range from 1.2 to 2.0 nm without any significant difference between CNFs derived from high and low lignin samples. However, there was a slight trend of reduced CNF height (thickness) from wood with the lowest lignin content (Fig. 2b, paper III). This tendency may be explained by cellulose and lignin competing for TEMPO oxidant and in case of low lignin content allowing

increased cellulose over lignin carboxylation. A higher carboxylation efficiency may thus give rise to finer CNF structures in wood samples with initial low lignin content.

Interestingly, the CNF diameter in both studies (paper II and III) were smaller than the diameter measured for elementary CMFs in native cell walls. Most likely, the small diameter of the isolated CNFs, derive from cleavage of the 3 nm elementary CMFs into smaller structures during the mechanical fibrillation process (Li and Renneckar, 2009, Usov *et al.*, 2015). The resolution for AFM is 0.1 nm which supports the statistical significance measured between TW and NW (Fig. 3, paper II). These observations raise the question about the origin of the very fine CNFs and about the native crystalline structure of the CMFs. The very thin CNFs possibly derive from a more loosely packed (less crystalline) CMF surface which would support the model that paracrystalline cellulose is located on the surface of the CMFs surrounding a highly crystalline core (Park *et al.*, 2010, Fernandes *et al.*, 2011). An alternative or additional theory would be that TEMPO attacks on the amorphous segments, hypothesized to interrupt highly crystalline cellulose regions (Atalla and VanderHart, 1984, Larsson *et al.*, 1997, Wickholm *et al.*, 1998) opening up the CMF structure from the inside as observed in some of the AFM images (Fig 4 and 5b, paper II).

Furthermore, it seems that in both studies (Paper II and III) the low lignin CNFs and TW CNFs exhibited the thinnest structures. This may support the idea that the other matrix polymers contribute to the thickness of CNFs. Even if it seems that the elementary CMFs are cleaved during fibrillation, and lignin is removed during TEMPO-mediated oxidation, it may be possible that lignin and/or hemicellulose, to some extent, remains on the surface and thus contribute to the thickness of the CNFs. This may explain the thinner CNFs obtained from TW and low lignin samples.

4.2.3 Mechanical properties of CNF networks

Mechanical properties of CNF networks were analyzed in order to investigate the effect of the raw material on the final quality of CNF networks. The mechanical properties of the CNFs networks are also of interested from a biological point of view since they may reflect the native mechanical wood properties.

TW CNFs networks showed significantly higher toughness and elongation at break values compared to NW CNFs (Fig. 9, paper II). Enhanced toughness of TW CNF networks may be associated with the higher cellulose DP in TW. In support of this idea cellulose DP and mechanical properties of nanocellulose have been shown to correlate (Henriksson *et al.*, 2008, Fukuzumi *et al.*, 2013). A higher DP is assumed to increase hydrogen bonds between both CMFs and CNFs, and thus support the cellulose network formation and contribute to a higher elongation before failure (Shinoda *et al.*, 2012, Zhu *et al.*, 2015). Compositional differences between CNF networks may also underline the differences in the mechanical properties. During CNF isolation, hemicellulose is not completely removed and comprises a significant proportion of the manufactured CNF networks. After removal of hemicellulose from CNF networks by using NaOH, CNF networks from TW contained ≤ 84 weight % cellulose while CNFs from NW contained ≤ 70 weight % cellulose (Fig. 10, paper II). Little is known about the effect of hemicellulose on mechanical properties of nanocellulose and some results are contradictory. One study reported that hemicellulose enhances the mechanical properties in CNF networks (Iwamoto *et al.*, 2008), while another study reported that the occurrence of hemicellulose in CNF networks reduces toughness and tensile strength (Arola *et al.*, 2013). We hypothesize the altered mechanical properties of TW CNF networks to derive from a combination of the hemicellulose content in CNF networks and the properties of CNFs.

In summary, the natural features of the G-layer, including highly crystalline cellulose with a high DP and low amounts and different composition of hemicellulose and almost no lignin, has a significant effect on the overall properties of TW derived CNFs. CNFs from TW were more difficult to fibrillate and CNFs were more heterogeneous size with thicker fibrils compared to CNFs from NW, most likely due to increased cellulose

crystallinity and DP. This is a clear drawback for the processing. On the other hand, TW CNFs showed enhanced mechanical performance apparent as increased toughness of the CNF networks which might be due to the structural and compositional features such as more recalcitrant microfibril arrangement (higher crystallinity) and higher DP (Foston *et al.*, 2011). In this context it is interesting to note that TW is less recalcitrant for enzymatic saccharification (Foston *et al.*, 2011, Sawada *et al.*, 2018). During saccharification, cellulose is broken down into single glucose units by performing enzymatic hydrolysis of the ß-1,4-linkages within the glucan chain. During CNF isolation, hydrogen bonds between individual CMFs are cleaved, while the ß-1,4-linkages are aimed to be preserved in order to keep the DP intact. Foston *et al.* (2011) hypothesized that an increased accessible cellulose surface in combination with higher cellulose and reduced lignin content are responsible for reduced recalcitrance in TW while increased cellulose crystallinity and DP may not be significant factors for saccharification efficiency. This highlights how different processing methods affect the cell wall and cellulose structure differently.

We observed no significant differences in specific strength, meaning the strength divided by the density of the CNF networks, with respect to lignin content of the starting material. The specific modulus differed between the low and high lignin wood derived CNF networks from 3 to 6 GPa while the elongation the elongation-at-break decreased from 8- 4 % with increasing lignin content (Fig. 6, paper III). This contradictory behavior may be explained by particles which were detected in CNF suspension derived from high lignin samples using AFM (Fig. 3, paper III). These particles may increase stiffness while decrease elongation at break and thus elasticity. No significant differences in the chemical composition and structure of the CNF networks, such as density, moisture content and thickness as well as porosity could be detected.

4.2.4 Wood porosity hypothesis

The improved fibrillation behavior shown for wood samples with high lignin content, are potentially associated with increased cell wall porosity after TEMPO-mediated oxidation. In order to investigate this hypothesis further, whole wood pieces with the lowest and highest lignin content respectively were directly treated with TEMPO and the surface area and pore size were analyzed as well as surface structure visualized by using SEM. Wood samples with initially high lignin content showed increased porosity compared to samples with initial low lignin content apparent as a larger surface area with 114 m^2g^{-1} for high lignin samples and 76 m^2g^{-1} for low lignin samples and an increased pore size in high lignin samples (5.0 nm) compared to low lignin samples (4.4 nm) (Table 3, paper III). SEM micrographs of TEMPO-oxidized wood pieces visually support the measured increased wood porosity in high lignin wood samples in both tangential and longitudinal wood sections (Fig. 7, paper III). We thus speculate that the increased wood porosity may relate to the improved fibrillation behavior of the high lignin samples by consequentially increasing cell wall porosity and thus making the cell wall structure more fragile for the mechanical fibrillation process.

4.3 Altered properties of cellulose nanofibrils from transgenic trees with reduced expression of *CELLULOSE SYNTHASE INTERACTING 1* (paper IV)

Wood from hybrid aspen trees with reduced expression of *CSI1* was used to study the effect of this genetical modification on CNF isolation and final properties. As described in **paper I**, the wood from the two *CSI1RNAi* lines (*CSI1RNAi-1* corresponds to T1 and *CSI1RNAi-3* corresponds to T2 **in paper IV**), were mechanically weaker and exhibited a reduction in cellulose DP compared to WT but without having differences in overall cell wall composition. Thus, we hypothesized that these changes may impact the purification process and quality of corresponding CNFs. As described before (**paper II and III**), CNFs were isolated by treating wood powder directly with TEMPO followed by mechanical fibrillation. The properties of the resulting CNF suspensions and networks are summarized and discussed in the following section.

4.3.1 CNF characterization

After TEMPO- mediated oxidation and subsequent mechanical fibrillation, there was no difference in the overall process yield, which for all samples ranged from 44-45 weight % of initial wood mass. At CNF suspension concentration above 0.20 weight %, viscosity was significantly higher for WT compared to both transgenic lines (Fig. 1d, paper IV) indicating a decreased fibrillation efficiency for both transgenic lines compared to WT. To investigate the origin of this difference, factors that are known to affect the viscosity, such as nanofibril yield, carboxylation content, and surface area, were measured.

The carboxylation content was significantly higher in WT (0.69 nmol/g) compared to both transgenic lines (0.55 nmol/g and 0.50 nm/g). Additionally, the nanofibril yield was slightly higher in WT (50.8 yield %) compared to both transgenic lines (49.1 and 48.2 yield %). Since there were no differences in the chemical composition and overall wood anatomy between the WT and both *CSI1RNAi* lines, the reduced carboxylation content

for the transgenic lines may relate to a reduced CMF accessibility for TEMPO-oxidation and subsequent fibrillation.

In support of this idea, the cellulosic surface area estimated by using Congo red was higher in WT compared to the transgenic lines with a reduction in surface area of approx. 20 m^2 g^{-1} or 20 %. These results agree with the reduced carboxylation content in the transgenic lines and also agree with previous studies which demonstrated that increased cellulosic surface area correlates with increased accessibility and thus fibrillation (Spence *et al*. 2011; Nge *et al*. 2013).

4.3.2 CNF network characterization

The cellulose DP from CNF networks was estimated by measuring the viscosity of dissolved CNF networks. The results indicate a significant difference between WT CNF networks and those from both transgenic lines (Fig. 4, paper IV). These results are interesting as they may align with the cellulose DP results obtained in paper I and support previous observations that the DP of CNFs is relatively well-preserved during TEMPO-oxidation and mechanical fibrillation and representative of the initial cellulose DP (Isogai *et al.*, 2009). Thus, we hypothesize that the differences in cellulose DP are preserved throughout the processing steps.

CNF networks derived from WT exhibited higher tensile strength and elongation-at-break compared to CNF networks derived from the transgenic lines (Fig. 3, paper IV). The Young´s modulus showed the opposite behavior and was lower for WT compared to the transgenic lines (Fig. 3c, paper IV). This means that CNF networks derived from WT are more flexible and resistant to stress and stretching while CNF networks derived from the transgenic lines are stiffer. Shorter CNFs were reported to result in more brittle and stiffer CNF network behavior (Henriksson *et al.*, 2008, Fukuzumi *et al.*, 2013, Meng *et al.*, 2017). Thus, the reduction in DP in CNF networks derived from both transgenic lines may explain the impaired mechanical performance measured in CNF networks from transgenic lines compared to WT. Thus, the differences in DP may have a major effect on the mechanical properties of the manufactured CNF networks.

The increased tensile strength and elongation-at-break for WT CNF networks compared to CNF networks from the transgenic lines also correlates with increased nanofibril yield, viscosity, degree of oxidation and surface area, which were reported to affect the mechanical network performance (Meng and Wang, 2019).

In this study, we could show that the reduced expression of *CSI1* in hybrid aspen wood had an effect on CNF isolation and final quality. The reduction of the native cellulose DP is reflected in the likewise reduced CNF-network DP obtained from both *CSI1RNAi* lines and contribute to the decreased mechanical strength and increased stiffness. The reduced accessibility of the CMFs apparent in parameters such as decreased nanofibril yield and viscosity may be due to structural changes of the cell wall matrix potentially caused by reduction in the DP. However, further investigation is required to dissect the origin of the CNF differences.

5. Conclusion and future perspectives

In an interdisciplinary approach this thesis project investigated the relevance of wood properties, cellulose biosynthesis and microfibril dimensions for nanocellulose production from aspen wood. In **paper I** showed that a reduction of *PtCSI1* causes impaired mechanical wood properties and a reduction in cellulose DP. I thus conclude that the reduction of cellulose DP is the main cause for the impaired mechanical wood properties, potentially in combination with additional structural changes in the SCW. Thus, this study further our understanding of CSI1 during cellulose biosynthesis, CMF alignment and wood mechanical properties and shows how the cell wall ultrastructure contribute to overall wood mechanical properties. As *CSI1RNAi* did not seem to affect the average cellulose MFA in the SCW, it would be interesting to measure cellulose orientation more precisely throughout the individual S-layers especially in the transition between PCW and S1-layer. This may help us to understand the underlying mechanism responsible for the changing cellulose orientation throughout the cell wall. Additionally, in order to study the role of CSI1 during wood formation in more depth, it would be interesting to create *csi-* null mutants using CRISPR-Cas-9 gene editing.

In **paper II** and **III,** we could show that native wood properties have an effect on the CNF isolation process as well as on the final performance and quality. TW, which contains due to the G-layer more highly crystalline cellulose with a higher cellulose DP and lower amounts of hemicellulose and lignin, was shown to be more difficult to process in order to obtain homogeneous CNFs. The decreased fibrillation efficiency was hypothesized to origin from the compositional and structural changes in the G-layer. However, an increased cellulose DP in TW was assumed to positively affect the mechanical

performance of manufactured CNF networks (**paper II)**. In **paper II,** we investigated the effect of native wood lignin content on CNF isolation by performing TEMPO-mediated oxidation. It was demonstrated that TEMPO, in addition to oxidizing cellulose, efficiently delignifies wood powder. Our results indicate that a high lignin content, counter intuitively, facilitates CNF isolation by improving the cellulose nanofibrillation process. We hypothesize that a TEMPO-mediated delignification causes increased cell wall porosity, making cellulose more accessible, and thus facilitating mechanical nanofibrillation. These results put lignin into a new light in terms of wood recalcitrance. Our results indicate that lignin reduces wood recalcitrance when using TEMPO-mediated oxidation and subsequent mechanical disintegration of the cell wall. Commonly, tree breeding aims to reduce or structurally modify lignin in order to reduce wood recalcitrance. However, these modifications often result in impaired tree growth, stress response and mechanical properties of the tree. Our results thus open new opportunities for tree breeding programs to improve the isolation of nanocellulose from wood. Due to the complexity of lignin and the effect on cell wall structure and overall tree growth, as well as on the nanocellulose extraction process, further investigations in this area are needed.

In paper IV we investigated suitability of hybrid aspen *CSI1RNAi* lines with impaired wood mechanical properties and a reduction in cellulose DP for CNF isolation and final CNF network properties. We discovered that cellulose from *CSI1RNAi* lines was more difficult to fibrillate resulting in a lower total CNF yield. We hypothesize that the reduced fibrillation efficiency is caused by structural changes of cellulose and/or cell wall matrix due to a reduction in DP in the transgenic lines. Interestingly, the initial reduction in cellulose DP as well as impaired mechanical properties were also apparent in CNF networks manufactured from both *CSI1RNAi* lines. It is thus possible that native cellulose and cell wall properties are reflected in the corresponding manufactured CNF networks. These results thus broaden our understanding about cell wall ultrastructure and the effect on CNF isolation and properties and opens possibilities to use genetically modified trees as a source for improved nanocellulose production.

Combined, these results of this thesis project demonstrate the importance of understanding the underlying structural and chemical composition of the cell wall for optimal CNF isolation efficiency and final performance. This in-depth knowledge is especially important for genetic breeding of wood properties for improved nanocellulose isolation.

References

Abe, H., Ohtani, J. and Fukazawa, K. (1991) FE-SEM observations on the microfibrillar orientation in the secondary wall of tracheids. *IAWA journal*, **12**, 431-438.

Abraham, Y. and Elbaum, R. (2013) Quantification of microfibril angle in secondary cell walls at subcellular resolution by means of polarized light microscopy. *New Phytologist*, **197**, 1012-1019.

Allen, H., Wei, D., Gu, Y. and Li, S. (2020) A historical perspective on the regulation of cellulose biosynthesis. *Carbohydrate Polymers*, 117022.

Anderson, C.T., Carroll, A., Akhmetova, L. and Somerville, C. (2010) Real-time imaging of cellulose reorientation during cell wall expansion in Arabidopsis roots. *Plant physiology*, **152**, 787-796.

Andersson, S., Serimaa, R., Paakkari, T., Saranpää, P. and Pesonen, E. (2003) Crystallinity of wood and the size of cellulose crystallites in Norway spruce (Picea abies). *Journal of Wood Science*, **49**, 531-537.

Arend, M. (2008) Immunolocalization of (1, 4)-β-galactan in tension wood fibers of poplar. *Tree Physiology*, **28**, 1263-1267.

Arioli, T., Peng, L., Betzner, A.S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Höfte, H., Plazinski, J. and Birch, R. (1998) Molecular analysis of cellulose biosynthesis in Arabidopsis. *Science*, **279**, 717-720.

Arola, S., Malho, J.M., Laaksonen, P., Lille, M. and Linder, M.B. (2013) The role of hemicellulose in nanofibrillated cellulose networks. *Soft Matter*, **9**, 1319-1326.

Atalla, R., Hackney, J., Uhlin, I. and Thompson, N. (1993) Hemicelluloses as structure regulators in the aggregation of native cellulose. *International journal of biological macromolecules*, **15**, 109-112.

Atalla, R.H. and VanderHart, D.L. (1984) Native cellulose: a composite of two distinct crystalline forms. *Science*, **223**, 283-286.

Bailey, I.W. and Kerr, T. (1935) The visible structure of the secondary wall and its significance in physical and chemical investigations of tracheary cells and fibers. *Journal of the Arnold Arboretum*, **16**, 273-300.

Barnett, J.R. and Bonham, V.A. (2004) Cellulose microfibril angle in the cell wall of wood fibres. *Biological reviews*, **79**, 461-472.

Bashline, L., Li, S. and Gu, Y. (2014) The trafficking of the cellulose synthase complex in higher plants. *Ann Bot*, **114**, 1059-1067.

Beery, W., Ifju, G. and McLain, T. (1983) Quantitative wood anatomy--relating anatomy to transverse tensile strength. *Wood and fiber science: journal of the Society of Wood Science and Technology*.

Begum, S., Nakaba, S., Yamagishi, Y., Oribe, Y. and Funada, R. (2013) Regulation of cambial activity in relation to environmental conditions: understanding the role of temperature in wood formation of trees. *Physiologia plantarum*, **147**, 46-54.

Ben-Tov, D., Idan-Molakandov, A., Hugger, A., Ben-Shlush, I., Günl, M., Yang, B., Usadel, B. and Harpaz-Saad, S. (2018) The role of COBRA-LIKE 2 function, as part of the complex network of interacting pathways regulating Arabidopsis seed mucilage polysaccharide matrix organization. *The Plant Journal*, **94**, 497-512.

Benedict, C., Kohel, R. and Jividen, G. (1994) Crystalline cellulose and cotton fiber strength. *Crop science*, **34**, 147-151.

Benfey, P.N., Linstead, P.J., Roberts, K., Schiefelbein, J.W., Hauser, M.-T. and Aeschbacher, R.A. (1993) Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis. *Development*, **119**, 57-70.

Bergander, A. and Salmén, L. (2002) Cell wall properties and their effects on the mechanical properties of fibers. *Journal of materials science*, **37**, 151-156.

Berglund, J., Mikkelsen, D., Flanagan, B.M., Dhital, S., Gaunitz, S., Henriksson, G., Lindström, M.E., Yakubov, G.E., Gidley, M.J. and Vilaplana, F. (2020) Wood hemicelluloses exert distinct biomechanical contributions to cellulose fibrillar networks. *Nature communications*, **11**, 1-16.

Biblis, E.J. (1969) Tensile properties of loblolly pine growth zones. *Wood and Fiber Science*, **1**, 18-28.

Biermann, O., Hädicke, E., Koltzenburg, S. and Müller-Plathe, F. (2001) Hydrophilicity and lipophilicity of cellulose crystal surfaces. *Angewandte Chemie International Edition*, **40**, 3822-3825.

Bjurhager, I., Olsson, A.-M., Zhang, B., Gerber, L., Kumar, M., Berglund, L.A., Burgert, I., Sundberg, B.r. and Salmén, L. (2010) Ultrastructure and mechanical properties of Populus wood with reduced lignin content caused by transgenic down-regulation of cinnamate 4-hydroxylase. *Biomacromolecules*, **11**, 2359-2365.

Boluk, Y., Moon, R., Ensor, D., Nieh, W., Forsstrom, U., Shatkin, J., Gardner, D., Teague, C., Haydon, B. and Walker, C. (2011) Roadmap for the Development of International Standards for Nanocellulose. *Arlington, US.*

Bonawitz, N.D. and Chapple, C. (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annual review of genetics*, **44**, 337-363.

Bondeson, D., Mathew, A. and Oksman, K. (2006) Optimization of the isolation of nanocrystals from microcrystalline cellulose by acid hydrolysis. *Cellulose*, **13**, 171-180.

Bonham, V. and Barnett, J. (2001) Fibre length and microfibril angle in silver birch (Betula pendula Roth).

Booker, R. and Sell, J. (1998) The nanostructure of the cell wall of softwoods and its functions in a living tree. *Holz als Roh-und Werkstoff*, **56**, 1.

Brändström, J., Bardage, S.L., Daniel, G. and Nilsson, T. (2003) The structural organisation of the S1 cell wall layer of Norway spruce tracheids. *IAWA journal*, **24**, 27-40.

Brett, C.T. (2000) Cellulose microfibrils in plants: biosynthesis, deposition, and integration into the cell wall. *International review of cytology*, **199**, 161-199.

Bringmann, M., Landrein, B., Schudoma, C., Hamant, O., Hauser, M.-T. and Persson, S. (2012a) Cracking the elusive alignment hypothesis: the microtubule–cellulose synthase nexus unraveled. *Trends in plant science*, **17**, 666-674.

Bringmann, M., Li, E., Sampathkumar, A., Kocabek, T., Hauser, M.T. and Persson, S. (2012b) POM-POM2/cellulose synthase interacting1 is essential for the functional association of cellulose synthase and microtubules in Arabidopsis. *Plant Cell*, **24**, 163-177.

Broxterman, S.E. and Schols, H.A. (2018) Interactions between pectin and cellulose in primary plant cell walls. *Carbohydrate polymers*, **192**, 263-272.

Burgert, I. (2006) Exploring the micromechanical design of plant cell walls. *American journal of botany*, **93**, 1391-1401.

Busse-Wicher, M., Gomes, T.C., Tryfona, T., Nikolovski, N., Stott, K., Grantham, N.J., Bolam, D.N., Skaf, M.S. and Dupree, P. (2014) The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of Arabidopsis thaliana. *The Plant Journal*, **79**, 492-506.

Carpita, N.C. and Gibeaut, D.M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, **3**, 1-30.

Cavalier, D.M., Lerouxel, O., Neumetzler, L., Yamauchi, K., Reinecke, A., Freshour, G., Zabotina, O.A., Hahn, M.G., Burgert, I. and Pauly, M. (2008) Disrupting two Arabidopsis thaliana xylosyltransferase genes results in plants deficient in xyloglucan, a major primary cell wall component. *The Plant Cell*, **20**, 1519-1537.

Cave, I. (1968) The anisotropic elasticity of the plant cell wall. *Wood science and technology*, **2**, 268-278.

Cave, I. (1969) The longitudinal Young's modulus of Pinus radiata. *Wood Science and Technology*, **3**, 40-48.

Cave, I. (1997) Theory of X-ray measurement of microfibril angle in wood. *Wood science and technology*, **31**, 225-234.

Cave, I. and Walker, J. (1994) Stiffness of wood in fast-grown plantation softwoods: the influence of microfibril angle. *Forest products journal*, **44**, 43.

Chafe, S. and Chauret, G. (1974) Cell wall structure in the xylem parenchyma of trembling aspen. *Protoplasma*, **80**, 129-147.

Chan, J. and Coen, E. (2020) Interaction between autonomous and microtubule guidance systems controls cellulose synthase trajectories. *Current Biology*, **30**, 941-947. e942.

Chen, Y.W., Lee, H.V., Juan, J.C. and Phang, S.-M. (2016) Production of new cellulose nanomaterial from red algae marine biomass Gelidium elegans. *Carbohydrate polymers*, **151**, 1210-1219.

Clair, B., Alméras, T., Pilate, G., Jullien, D., Sugiyama, J. and Riekel, C. (2010) Maturation stress generation in poplar tension wood studied by synchrotron radiation microdiffraction. *Plant physiology*, **152**, 1650-1658.

Coleman, H.D., Park, J.-Y., Nair, R., Chapple, C. and Mansfield, S.D. (2008) RNAi-mediated suppression of p-coumaroyl-CoA 3′-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism. *Proceedings of the National Academy of Sciences*, **105**, 4501-4506.

Cosgrove, D.C. (2012) Comparative structure and biomechanics of plant primary and secondary cell walls. *Frontiers in plant science*, **3**, 204.

Cosgrove, D.J. (2005) Growth of the plant cell wall. *Nature reviews molecular cell biology*, **6**, 850.

Cousins, W. (1976) Elastic modulus of lignin as related to moisture content. *Wood science and technology*, **10**, 9-17.

Cuny, H.E., Rathgeber, C.B., Frank, D., Fonti, P. and Fournier, M. (2014) Kinetics of tracheid development explain conifer tree-ring structure. *New Phytologist*, **203**, 1231-1241.

Dadswell, H. and Wardrop, A. (1955) The structure and properties of tension wood.

Déjardin, A., Laurans, F., Arnaud, D., Breton, C., Pilate, G. and Leplé, J.-C. (2010) Wood formation in Angiosperms. *Comptes rendus biologies*, **333**, 325-334.

Derba-Maceluch, M., Awano, T., Takahashi, J., Lucenius, J., Ratke, C., Kontro, I., Busse-Wicher, M., Kosik, O., Tanaka, R. and Winzéll, A. (2015) Suppression of xylan endotransglycosylase PtxtXyn10A affects cellulose microfibril angle in secondary wall in aspen wood. *New Phytologist*, **205**, 666-681.

Desprez, T., Juraniec, M., Crowell, E.F., Jouy, H., Pochylova, Z., Parcy, F., Höfte, H., Gonneau, M. and Vernhettes, S. (2007) Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, **104**, 15572-15577.

Dhali, K., Ghasemlou, M., Daver, F., Cass, P. and Adhikari, B. (2021) A review of nanocellulose as a new material towards environmental sustainability. *Science of the Total Environment*, 145871.

Ding, S.-Y. and Himmel, M.E. (2006) The maize primary cell wall microfibril: a new model derived from direct visualization. *Journal of agricultural and food chemistry*, **54**, 597-606.

Donaldson, L. (2007) Cellulose microfibril aggregates and their size variation with cell wall type. *Wood science and technology*, **41**, 443-460.
Donaldson, L. (2008) Microfibril angle: measurement, variation and relationships–a review. *Iawa Journal*, **29**, 345-386.
Donaldson, L.A. and Knox, J.P. (2012) Localization of cell wall polysaccharides in normal and compression wood of radiata pine: relationships with lignification and microfibril orientation. *Plant Physiology*, **158**, 642-653.
Dufresne, A. (2013) Nanocellulose: a new ageless bionanomaterial. *Materials today*, **16**, 220-227.
Dufresne, A. (2017a) Cellulose nanomaterial reinforced polymer nanocomposites. *Current Opinion in Colloid & Interface Science*, **29**, 1-8.
Dufresne, A. (2017b) *Nanocellulose: from nature to high performance tailored materials*: Walter de Gruyter GmbH & Co KG.
Dufresne, A. (2019) Nanocellulose processing properties and potential applications. *Current Forestry Reports*, **5**, 76-89.
Ek, M., Gellerstedt, G. and Henriksson, G. (2009) *Wood chemistry and biotechnology*: Walter de Gruyter.
Evans, R. (1999) A variance approach to the X-ray diffractometric estimation of microfibril angle in wood. *Appita Journal*, **52**, 283-289.
Evans, R. and Elic, J. (2001) Rapid prediction of wood stiffness from microfibril angle and density. *Forest products journal*, **51**.
Evans, R., Stringer, S. and Kibblewhite, R.P. (2000) Variation of microfibril angle, density and fibre orientation in twenty-nine Eucalyptus nitens trees. *Appita Journal*, **53**, 450-457.
Fahlén, J. and Salmén, L. (2005) Pore and matrix distribution in the fiber wall revealed by atomic force microscopy and image analysis. *Biomacromolecules*, **6**, 433-438.
Fahn, A. (1967) Plant anatomy. *Plant anatomy.*
Fan, D., Liu, T., Li, C., Jiao, B., Li, S., Hou, Y. and Luo, K. (2015) Efficient CRISPR/Cas9-mediated Targeted Mutagenesis in Populus in the First Generation. *Sci Rep*, **5**, 12217.
Felten, J. and Sundberg, B. (2013) Biology, Chemistry and Structure of Tension Wood. **20**, 203-224.
Fengel, D. and Stoll, M. (1973) Über die Veränderungen des Zellquerschnitts, der Dicke der Zellwand und der Wandschichten von Fichtenholz-Tracheiden innerhalb eines Jahrringes.
Fernandes, A.N., Thomas, L.H., Altaner, C.M., Callow, P., Forsyth, V.T., Apperley, D.C., Kennedy, C.J. and Jarvis, M.C. (2011) Nanostructure of cellulose microfibrils in spruce wood. *Proceedings of the National Academy of Sciences*, **108**, E1195-E1203.
Feynman, R.P. (1961) There's plenty of room at the bottom. *Miniaturization*, **282**, 295-296.
Filipova, I., Serra, F., Tarres, Q., Mutje, P. and Delgado-Aguilar, M. (2020) Oxidative treatments for cellulose nanofibers production: a comparative

study between TEMPO-mediated and ammonium persulfate oxidation. *Cellulose*, **27**.

Filson, P.B. and Dawson-Andoh, B.E. (2009) Sono-chemical preparation of cellulose nanocrystals from lignocellulose derived materials. *Bioresource Technology*, **100**, 2259-2264.

Foston, M., Hubbell, C.A., Samuel, R., Jung, S., Fan, H., Ding, S.-Y., Zeng, Y., Jawdy, S., Davis, M. and Sykes, R. (2011) Chemical, ultrastructural and supramolecular analysis of tension wood in Populus tremula x alba as a model substrate for reduced recalcitrance. *Energy & Environmental Science*, **4**, 4962-4971.

Fu, Y., Gu, Y., Zheng, Z., Wasteneys, G. and Yang, Z. (2005) Arabidopsis interdigitating cell growth requires two antagonistic pathways with opposing action on cell morphogenesis. *Cell*, **120**, 687-700.

Fujikawa, S. (1975) Ultrastructure of ray parenchyma cell wall of softwood.

Fukuzumi, H., Saito, T. and Isogai, A. (2013) Influence of TEMPO-oxidized cellulose nanofibril length on film properties. *Carbohydrate Polymers*, **93**, 172-177.

Funada, R., Abe, H., Furusawa, O., Imaizumi, H., Fukazawa, K. and Ohtani, J. (1997) The orientation and localization of cortical microtubules in differentiating conifer tracheids during cell expansion. *Plant and cell physiology*, **38**, 210-212.

Gardner, K. and Blackwell, J. (1974) The structure of native cellulose. *Biopolymers: Original Research on Biomolecules*, **13**, 1975-2001.

Gibson, L.J. (2012) The hierarchical structure and mechanics of plant materials. *Journal of the royal society interface*, **9**, 2749-2766.

Gonneau, M., Desprez, T., Guillot, A., Vernhettes, S. and Höfte, H. (2014) Catalytic subunit stoichiometry within the cellulose synthase complex. *Plant physiology*, **166**, 1709-1712.

Gorshkova, T., Brutch, N., Chabbert, B., Deyholos, M., Hayashi, T., Lev-Yadun, S., Mellerowicz, E.J., Morvan, C., Neutelings, G. and Pilate, G. (2012) Plant fiber formation: state of the art, recent and expected progress, and open questions. *Critical Reviews in Plant Sciences*, **31**, 201-228.

Gorshkova, T., Mokshina, N., Chernova, T., Ibragimova, N., Salnikov, V., Mikshina, P., Tryfona, T., Banasiak, A., Immerzeel, P., Dupree, P. and Mellerowicz, E.J. (2015) Aspen Tension Wood Fibers Contain beta-(1---> 4)-Galactans and Acidic Arabinogalactans Retained by Cellulose Microfibrils in Gelatinous Walls. *Plant Physiol*, **169**, 2048-2063.

Goswami, L., Dunlop, J.W., Jungnikl, K., Eder, M., Gierlinger, N., Coutand, C., Jeronimidis, G., Fratzl, P. and Burgert, I. (2008) Stress generation in the tension wood of poplar is based on the lateral swelling power of the G-layer. *Plant J*, **56**, 531-538.

Grantham, N.J., Wurman-Rodrich, J., Terrett, O.M., Lyczakowski, J.J., Stott, K., Iuga, D., Simmons, T.J., Durand-Tardif, M., Brown, S.P. and

Dupree, R. (2017) An even pattern of xylan substitution is critical for interaction with cellulose in plant cell walls. *Nature plants*, **3**, 859-865.

Green, P.B. (1962) Mechanism for plant cellular morphogenesis. *Science*, **138**, 1404-1405.

Groom, L., Shaler, S. and Mott, L. (2002) Mechanical properties of individual southern pine fibers. Part III: Global relationships between fiber properties and fiber location within an individual tree. *Wood and Fiber Science*, **34**, 238-250.

Gu, Y., Kaplinsky, N., Bringmann, M., Cobb, A., Carroll, A., Sampathkumar, A., Baskin, T.I., Persson, S. and Somerville, C.R. (2010) Identification of a cellulose synthase-associated protein required for cellulose biosynthesis. *Proceedings of the National Academy of Sciences*, **107**, 12866-12871.

Habibi, Y., Lucia, L.A. and Rojas, O.J. (2010) Cellulose nanocrystals: chemistry, self-assembly, and applications. *Chemical reviews*, **110**, 3479-3500.

Hancock, J.E., Loya, W.M., Giardina, C.P., Li, L., Chiang, V.L. and Pregitzer, K.S. (2007) Plant growth, biomass partitioning and soil carbon formation in response to altered lignin biosynthesis in Populus tremuloides. *New Phytologist*, **173**, 732-742.

Harada, H. and Côté, W. (1985) Structure of wood. *Biosynthesis and biodegradation of wood components*, **1**, 1-42.

Henriksson, M., Berglund, L.A., Isaksson, P., Lindstrom, T. and Nishino, T. (2008) Cellulose nanopaper structures of high toughness. *Biomacromolecules*, **9**, 1579-1585.

Hermans, P., de Booys, J. and Maan, C.J. (1943) Über die Gestalt und die Beweglichkeit des Moleküls der Zellulose. *Kolloid-Zeitschrift*, **102**, 169-180.

Herth, W. (1983) Arrays of plasma-membrane "rosettes" involved in cellulose microfibril formation of Spirogyra. *Planta*, **159**, 347-356.

Hill, J.L., Hammudi, M.B. and Tien, M. (2014) The Arabidopsis cellulose synthase complex: a proposed hexamer of CESA trimers in an equimolar stoichiometry. *The Plant Cell*, **26**, 4834-4842.

Hodge, A. and Wardrop, A. (1950) An electron microscopic investigation of the cell wall organization of conifer tracheids and conifer cambium. *Australian Journal of Biological Sciences*, **3**, 265-269.

Horvath, L., Peszlen, I., Peralta, P., Kasal, B. and Li, L. (2010) Mechanical properties of genetically engineered young aspen with modified lignin content and/or structure. *Wood and Fiber Science*, **42**, 310-317.

Houtman, C.J. and Atalla, R.H. (1995) Cellulose-lignin interactions (a computational study). *Plant physiology*, **107**, 977-984.

Hubbell, C.A. and Ragauskas, A.J. (2010) Effect of acid-chlorite delignification on cellulose degree of polymerization. *Bioresource technology*, **101**, 7410-7415.

Huntley, S.K., Ellis, D., Gilbert, M., Chapple, C. and Mansfield, S.D. (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: improved chemical savings and reduced environmental toxins. *Journal of Agricultural and Food Chemistry*, **51**, 6178-6183.

Isogai, A. (2013) Wood nanocelluloses: fundamentals and applications as new bio-based nanomaterials. *Journal of Wood Science*, **59**, 449-459.

Isogai, A. (2021) Emerging nanocellulose technologies: Recent developments. *Advanced Materials*, **33**, 2000630.

Isogai, A., Saito, T. and Fukuzumi, H. (2011) TEMPO-oxidized cellulose nanofibers. *nanoscale*, **3**, 71-85.

Isogai, T., Yanagisawa, M. and Isogai, A. (2009) Degrees of polymerization (DP) and DP distribution of cellouronic acids prepared from alkali-treated celluloses and ball-milled native celluloses by TEMPO-mediated oxidation. *Cellulose*, **16**, 117-127.

Iwamoto, S., Abe, K. and Yano, H. (2008) The effect of hemicelluloses on wood pulp nanofibrillation and nanofiber network characteristics. *Biomacromolecules*, **9**, 1022-1026.

Jansson, S. and Douglas, C.J. (2007) Populus: a model system for plant biology. *Annu. Rev. Plant Biol.*, **58**, 435-458.

Jiang, F., Esker, A.R. and Roman, M. (2010) Acid-catalyzed and solvolytic desulfation of H2SO4-hydrolyzed cellulose nanocrystals. *Langmuir*, **26**, 17919-17925.

Jin, X.-J. and Pascal Kamdem, D. (2009) Chemical composition, crystallinity and crystallite cellulose size in populus hybrids and aspen. *Cellulose Chemistry & Technology*, **43**, 229.

Jonasson, S., Bünder, A., Niittylä, T. and Oksman, K. (2020) Isolation and characterization of cellulose nanofibers from aspen wood using derivatizing and non-derivatizing pretreatments. *Cellulose*, **27**, 185-203.

Jonoobi, M., Mathew, A.P. and Oksman, K. (2012) Producing low-cost cellulose nanofiber from sludge as new source of raw materials. *Industrial Crops and Products*, **40**, 232-238.

Jonoobi, M., Oladi, R., Davoudpour, Y., Oksman, K., Dufresne, A., Hamzeh, Y. and Davoodi, R. (2015) Different preparation methods and properties of nanostructured cellulose from various natural resources and residues: a review. *Cellulose*, **22**, 935-969.

Joseleau, J.-P., Imai, T., Kuroda, K. and Ruel, K. (2004) Detection in situ and characterization of lignin in the G-layer of tension wood fibres of Populus deltoides. *Planta*, **219**, 338-345.

Jozala, A.F., de Lencastre-Novaes, L.C., Lopes, A.M., de Carvalho Santos-Ebinuma, V., Mazzola, P.G., Pessoa-Jr, A., Grotto, D., Gerenutti, M. and Chaud, M.V. (2016) Bacterial nanocellulose production and application: a 10-year overview. *Applied microbiology and biotechnology*, **100**, 2063-2072.

Jozsa, L. and Middleton, G. (1994) A discussion of wood quality attributes and their practical implications.

Kaneda, M., Rensing, K. and Samuels, L. (2010) Secondary cell wall deposition in developing secondary xylem of poplar. *Journal of integrative plant biology*, **52**, 234-243.

Kang, X., Kirui, A., Widanage, M.C.D., Mentink-Vigier, F., Cosgrove, D.J. and Wang, T. (2019) Lignin-polysaccharide interactions in plant secondary cell walls revealed by solid-state NMR. *Nature communications*, **10**, 1-9.

Klemm, D., Cranston, E.D., Fischer, D., Gama, M., Kedzior, S.A., Kralisch, D., Kramer, F., Kondo, T., Lindström, T. and Nietzsche, S. (2018) Nanocellulose as a natural source for groundbreaking applications in materials science: Today's state. *Materials Today*, **21**, 720-748.

Klemm, D., Schumann, D., Kramer, F., Heßler, N., Koth, D. and Sultanova, B. (2009) Nanocellulose materials–different cellulose, different functionality. In *Macromolecular symposia*: Wiley Online Library, pp. 60-71.

Köhler, L. and Spatz, H.-C. (2002) Micromechanics of plant tissues beyond the linear-elastic range. *Planta*, **215**, 33-40.

Kostoff, R.N., Koytcheff, R.G. and Lau, C.G. (2007) Global nanotechnology research literature overview. *Technological forecasting and social change*, **74**, 1733-1747.

Kretschmann, D.E. (2010) Wood handbook, chapter 05: mechanical properties of wood. *Forest Products Laboratory, Department of Agriculture Forest Service, Madison, Wisconsin, USA*.

Kumar, M., Campbell, L. and Turner, S. (2016) Secondary cell walls: biosynthesis and manipulation. *Journal of experimental botany*, **67**, 515-531.

Kumar, R., Mago, G., Balan, V. and Wyman, C.E. (2009) Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource technology*, **100**, 3948-3962.

Landrein, B., Lathe, R., Bringmann, M., Vouillot, C., Ivakov, A., Boudaoud, A., Persson, S. and Hamant, O. (2013) Impaired cellulose synthase guidance leads to stem torsion and twists phyllotactic patterns in Arabidopsis. *Current Biology*, **23**, 895-900.

Larson, P.R. (1994) Defining the cambium. In *The Vascular Cambium*: Springer, pp. 33-97.

Larson, P.R. (2012) *The vascular cambium: development and structure*: Springer Science & Business Media.

Larsson, P.T., Wickholm, K. and Iversen, T. (1997) A CP/MAS13C NMR investigation of molecular ordering in celluloses. *Carbohydrate Research*, **302**, 19-25.

Lautner, S., Zollfrank, C. and Fromm, J. (2012) Microfibril angle distribution of poplar tension wood. *Iawa Journal*, **33**, 431-439.

Ledbetter, M. and Porter, K. (1963) A" microtubule" in plant cell fine structure. *The Journal of cell biology*, **19**, 239-250.

Lei, L., Li, S., Du, J., Bashline, L. and Gu, Y. (2013) Cellulose synthase INTERACTIVE3 regulates cellulose biosynthesis in both a microtubule-dependent and microtubule-independent manner in Arabidopsis. *The Plant Cell*, **25**, 4912-4923.

Lei, L., Singh, A., Bashline, L., Li, S., Yingling, Y.G. and Gu, Y. (2015) CELLULOSE SYNTHASE INTERACTIVE1 Is Required for Fast Recycling of Cellulose Synthase Complexes to the Plasma Membrane in Arabidopsis. *Plant Cell*, **27**, 2926-2940.

Leu, S.-Y. and Zhu, J. (2013) Substrate-related factors affecting enzymatic saccharification of lignocelluloses: our recent understanding. *Bioenergy Research*, **6**, 405-415.

Li, M., Pu, Y. and Ragauskas, A.J. (2016a) Current understanding of the correlation of lignin structure with biomass recalcitrance. *Frontiers in chemistry*, **4**, 45.

Li, Q. and Renneckar, S. (2009) Molecularly thin nanoparticles from cellulose: isolation of sub-microfibrillar structures. *Cellulose*, **16**, 1025-1032.

Li, S., Bashline, L., Zheng, Y., Xin, X., Huang, S., Kong, Z., Kim, S.H., Cosgrove, D.J. and Gu, Y. (2016b) Cellulose synthase complexes act in a concerted fashion to synthesize highly aggregated cellulose in secondary cell walls of plants. *Proc Natl Acad Sci U S A*, **113**, 11348-11353.

Li, S., Lei, L., Somerville, C.R. and Gu, Y. (2012) Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. *Proceedings of the National Academy of Sciences*, **109**, 185-190.

Lichtenegger, H., Reiterer, A., Stanzl-Tschegg, S. and Fratzl, P. (1999) Variation of cellulose microfibril angles in softwoods and hardwoods—a possible strategy of mechanical optimization. *Journal of structural biology*, **128**, 257-269.

Liu, Z., Schneider, R., Kesten, C., Zhang, Y., Somssich, M., Zhang, Y., Fernie, A.R. and Persson, S. (2016) Cellulose-microtubule uncoupling proteins prevent lateral displacement of microtubules during cellulose synthesis in Arabidopsis. *Developmental Cell*, **38**, 305-315.

Lyczakowski, J.J., Bourdon, M., Terrett, O.M., Helariutta, Y., Wightman, R. and Dupree, P. (2019) Structural imaging of native cryo-preserved secondary cell walls reveals the presence of macrofibrils and their formation requires normal cellulose, lignin and xylan biosynthesis. *Frontiers in plant science*, **10**, 1398.

Ma, P., Fu, S., Zhai, H., Law, K. and Daneault, C. (2012) Influence of TEMPO-mediated oxidation on the lignin of thermomechanical pulp. *Bioresource technology*, **118**, 607-610.

Mania, P., Siuda, F. and Roszyk, E. (2020) Effect of slope grain on mechanical properties of different wood species. *Materials*, **13**, 1503.

Mansfield, S.D., Kang, K.Y. and Chapple, C. (2012) Designed for deconstruction–poplar trees altered in cell wall lignification improve the efficacy of bioethanol production. *New Phytologist*, **194**, 91-101.

Mellerowicz, E.J., Baucher, M., Sundberg, B. and Boerjan, W. (2001) Unravelling cell wall formation in the woody dicot stem. *Plant cell walls*, 239-274.

Mellerowicz, E.J. and Sundberg, B. (2008) Wood cell walls: biosynthesis, developmental dynamics and their implications for wood properties. *Current opinion in plant biology*, **11**, 293-300.

Meng, Q., Li, B., Li, T. and Feng, X.-Q. (2017) A multiscale crack-bridging model of cellulose nanopaper. *Journal of the Mechanics and Physics of Solids*, **103**, 22-39.

Meng, Q. and Wang, T.J. (2019) Mechanics of strong and tough cellulose nanopaper. *applied mechanics reviews*, **71**.

Meyer, K.H. and Misch, L. (1937) Positions des atomes dans le nouveau modele spatial de la cellulose. *Helvetica Chimica Acta*, **20**, 232-244.

Miedes, E., Vanholme, R., Boerjan, W. and Molina, A. (2014) The role of the secondary cell wall in plant resistance to pathogens. *Frontiers in plant science*, **5**, 358.

Moon, R.J., Martini, A., Nairn, J., Simonsen, J. and Youngblood, J. (2011) Cellulose nanomaterials review: structure, properties and nanocomposites. *Chemical Society Reviews*, **40**, 3941-3994.

Moura, J.C.M.S., Bonine, C.A.V., de Oliveira Fernandes Viana, J., Dornelas, M.C. and Mazzafera, P. (2010) Abiotic and biotic stresses and changes in the lignin content and composition in plants. *Journal of integrative plant biology*, **52**, 360-376.

Mueller, S.C. and Brown Jr, R.M. (1980) Evidence for an intramembrane component associated with a cellulose microfibril-synthesizing complex in higher plants. *J. Cell Biol*, **84**, 315-326.

Müller, M., Burghammer, M. and Sugiyama, J. (2006) Direct investigation of the structural properties of tension wood cellulose microfibrils using microbeam X-ray fibre diffraction. *Holzforschung*, **60**, 474-479.

Nägeli, C. (1858) *Pflanzenphysiologische Untersuchungen*: Schulthess.

Nakaba, S., Begum, S., Yamagishi, Y., Jin, H.-O., Kubo, T. and Funada, R. (2012) Differences in the timing of cell death, differentiation and function among three different types of ray parenchyma cells in the hardwood Populus sieboldii× P. grandidentata. *Trees*, **26**, 743-750.

Newman, R.H., Hill, S.J. and Harris, P.J. (2013) Wide-angle x-ray scattering and solid-state nuclear magnetic resonance data combined to test models for cellulose microfibrils in mung bean cell walls. *Plant physiology*, **163**, 1558-1567.

Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H. and Höfte, H. (1998) A plasma membrane-bound putative endo-1, 4-β-d-glucanase is required for normal wall assembly and cell elongation in Arabidopsis. *The EMBO journal*, **17**, 5563-5576.

Nieduszynski, I. and Marchessault, R. (1971) Structure of β-D-(1→ 4′) xylan hydrate. *Nature*, **232**, 46-47.

Nishikubo, N., Awano, T., Banasiak, A., Bourquin, V., Ibatullin, F., Funada, R., Brumer, H., Teeri, T.T., Hayashi, T. and Sundberg, B. (2007) Xyloglucan endo-transglycosylase (XET) functions in gelatinous layers of tension wood fibers in poplar—a glimpse into the mechanism of the balancing act of trees.

Nishiyama, Y., Langan, P. and Chanzy, H. (2002) Crystal structure and hydrogen-bonding system in cellulose Iβ from synchrotron X-ray and neutron fiber diffraction. *Journal of the American Chemical Society*, **124**, 9074-9082.

Norberg, P.H. and Meier, H. (1966) Physical and chemical properties of the gelatinous layer in tension wood fibres of aspen (Populus tremula L.). *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, **20**, 174-178.

Özparpucu, M., Rüggeberg, M., Gierlinger, N., Cesarino, I., Vanholme, R., Boerjan, W. and Burgert, I. (2017) Unravelling the impact of lignin on cell wall mechanics: a comprehensive study on young poplar trees downregulated for CINNAMYL ALCOHOL DEHYDROGENASE (CAD). *The Plant Journal*, **91**, 480-490.

Pallardy, S.G. (2010) *Physiology of woody plants*: academic press.

Paredez, A.R., Persson, S., Ehrhardt, D.W. and Somerville, C.R. (2008) Genetic evidence that cellulose synthase activity influences microtubule cortical array organization. *Plant physiology*, **147**, 1723-1734.

Paredez, A.R., Somerville, C.R. and Ehrhardt, D.W. (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. *Science*, **312**, 1491-1495.

Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A. and Johnson, D.K. (2010) Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for biofuels*, **3**, 10.

Park, Y.B. and Cosgrove, D.J. (2012) A revised architecture of primary cell walls based on biomechanical changes induced by substrate-specific endoglucanases. *Plant Physiology*, **158**, 1933-1943.

Park, Y.B. and Cosgrove, D.J. (2015) Xyloglucan and its interactions with other components of the growing cell wall. *Plant and Cell Physiology*, **56**, 180-194.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M. and Somerville, C.R. (2007) Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in Arabidopsis. *Proceedings of the National Academy of Sciences*, **104**, 15566-15571.

Phanthong, P., Reubroycharoen, P., Hao, X., Xu, G., Abudula, A. and Guan, G. (2018) Nanocellulose: Extraction and application. *Carbon Resources Conversion*, **1**, 32-43.

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leplé, J.-C., Pollet, B., Mila, I., Webster, E.A. and Marstorp, H.G. (2002) Field and pulping

performances of transgenic trees with altered lignification. *Nature biotechnology*, **20**, 607-612.

Plomion, C., Leprovost, G. and Stokes, A. (2001) Wood formation in trees. *Plant physiology*, **127**, 1513-1523.

Pope, D., Marcroft, J. and Whale, L. (2005) The effect of global slope of grain on the bending strength of scaffold boards. *Holz als Roh-und Werkstoff*, **63**, 321-326.

Preston, R. (1979) Polysaccharide conformation and cell wall function. *Annual Review of Plant Physiology*, **30**, 55-78.

Preston, R.D. (1974) *The physical biology of plant cell walls*: London.: Chapman & Hall.

Purushotham, P., Cho, S.H., Diaz-Moreno, S.M., Kumar, M., Nixon, B.T., Bulone, V. and Zimmer, J. (2016) A single heterologously expressed plant cellulose synthase isoform is sufficient for cellulose microfibril formation in vitro. *Proc Natl Acad Sci U S A*, **113**, 11360-11365.

Reza, M., Bertinetto, C., Kesari, K.K., Engelhardt, P., Ruokolainen, J. and Vuorinen, T. (2019) Cellulose elementary fibril orientation in the spruce S 1-2 transition layer. *Scientific reports*, **9**, 1-7.

Roduner, E. (2006) Size matters: why nanomaterials are different. *Chemical Society Reviews*, **35**, 583-592.

Rongpipi, S., Ye, D., Gomez, E.D. and Gomez, E.W. (2019) Progress and opportunities in the characterization of cellulose–an important regulator of cell wall growth and mechanics. *Frontiers in plant science*, **9**, 1894.

Roudier, F., Fernandez, A.G., Fujita, M., Himmelspach, R., Borner, G.H., Schindelman, G., Song, S., Baskin, T.I., Dupree, P. and Wasteneys, G.O. (2005) COBRA, an Arabidopsis extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. *The Plant Cell*, **17**, 1749-1763.

Roudier, F., Schindelman, G., DeSalle, R. and Benfey, P.N. (2002) The COBRA family of putative GPI-anchored proteins in Arabidopsis. A new fellowship in expansion. *Plant Physiology*, **130**, 538-548.

Rüggeberg, M., Saxe, F., Metzger, T.H., Sundberg, B., Fratzl, P. and Burgert, I. (2013) Enhanced cellulose orientation analysis in complex model plant tissues. *Journal of structural biology*, **183**, 419-428.

Saito, T., Kimura, S., Nishiyama, Y. and Isogai, A. (2007) Cellulose nanofibers prepared by TEMPO-mediated oxidation of native cellulose. *Biomacromolecules*, **8**, 2485-2491.

Saito, T., Nishiyama, Y., Putaux, J.-L., Vignon, M. and Isogai, A. (2006) Homogeneous suspensions of individualized microfibrils from TEMPO-catalyzed oxidation of native cellulose. *Biomacromolecules*, **7**, 1687-1691.

Sampathkumar, A., Krupinski, P., Wightman, R., Milani, P., Berquand, A., Boudaoud, A., Hamant, O., Jönsson, H. and Meyerowitz, E.M. (2014)

Subcellular and supracellular mechanical stress prescribes cytoskeleton behavior in Arabidopsis cotyledon pavement cells. *Elife*, **3**, e01967.

Sarén, M.-P. and Serimaa, R. (2006) Determination of microfibril angle distribution by X-ray diffraction. *Wood Science and Technology*, **40**, 445.

Sawada, D., Kalluri, U.C., O'Neill, H., Urban, V., Langan, P., Davison, B. and Pingali, S.V. (2018) Tension wood structure and morphology conducive for better enzymatic digestion. *Biotechnology for biofuels*, **11**, 1-9.

Scheller, H.V. and Ulvskov, P. (2010) Hemicelluloses. *Annual review of plant biology*, **61**.

Schneider, R., Tang, L., Lampugnani, E.R., Barkwill, S., Lathe, R., Zhang, Y., McFarlane, H.E., Pesquet, E., Niittyla, T. and Mansfield, S.D. (2017) Two complementary mechanisms underpin cell wall patterning during xylem vessel development. *The Plant Cell*, **29**, 2433-2449.

Schuetz, M., Smith, R. and Ellis, B. (2013) Xylem tissue specification, patterning, and differentiation mechanisms. *Journal of experimental botany*, **64**, 11-31.

Shaw, S.L., Kamyar, R. and Ehrhardt, D.W. (2003) Sustained microtubule treadmilling in Arabidopsis cortical arrays. *Science*, **300**, 1715-1718.

Shinoda, R., Saito, T., Okita, Y. and Isogai, A. (2012) Relationship between length and degree of polymerization of TEMPO-oxidized cellulose nanofibrils. *Biomacromolecules*, **13**, 842-849.

Simmons, T.J., Mortimer, J.C., Bernardinelli, O.D., Pöppler, A.-C., Brown, S.P., Deazevedo, E.R., Dupree, R. and Dupree, P. (2016) Folding of xylan onto cellulose fibrils in plant cell walls revealed by solid-state NMR. *Nature communications*, **7**, 1-9.

Sjöström, E. (1993) Wood polysaccharides. *Wood chemistry, fundamentals and applications*, 51-70.

Somerville, C. (2006) Cellulose synthesis in higher plants. *Annu Rev Cell Dev Biol*, **22**, 53-78.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S. and Raab, T. (2004) Toward a systems approach to understanding plant cell walls. *Science*, **306**, 2206-2211.

Sorieul, M., Dickson, A., Hill, S.J. and Pearson, H. (2016) Plant fibre: molecular structure and biomechanical properties, of a complex living material, influencing its deconstruction towards a biobased composite. *Materials*, **9**, 618.

Spicer, R. and Groover, A. (2010) Evolution of development of vascular cambia and secondary growth. *New Phytologist*, **186**, 577-592.

Staudinger, H. (1934) Die Chemie der Cellulose. *Naturwissenschaften*, **22**, 797-803.

Stuart, S.-A. and Evans, R. (1994) *X-ray diffraction estimation of the microfibril angle variation in eucalypt increment cores*: CRC Publications Committee.

Takahashi, J., Rudsander, U.J., Hedenström, M., Banasiak, A., Harholt, J., Amelot, N., Immerzeel, P., Ryden, P., Endo, S. and Ibatullin, F.M. (2009) KORRIGAN1 and its aspen homolog PttCel9A1 decrease cellulose crystallinity in Arabidopsis stems. *Plant and cell physiology*, **50**, 1099-1115.

Takata, N., Awano, T., Nakata, M.T., Sano, Y., Sakamoto, S., Mitsuda, N. and Taniguchi, T. (2019) Populus NST/SND orthologs are key regulators of secondary cell wall formation in wood fibers, phloem fibers and xylem ray parenchyma cells. *Tree physiology*, **39**, 514-525.

Taylor, N.G., Howells, R.M., Huttly, A.K., Vickers, K. and Turner, S.R. (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proceedings of the National Academy of Sciences*, **100**, 1450-1455.

Taylor, N.G., Scheible, W.-R., Cutler, S., Somerville, C.R. and Turner, S.R. (1999) The irregular xylem3 locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *The plant cell*, **11**, 769-779.

Thomas, L.H., Forsyth, V.T., Šturcová, A., Kennedy, C.J., May, R.P., Altaner, C.M., Apperley, D.C., Wess, T.J. and Jarvis, M.C. (2013) Structure of cellulose microfibrils in primary cell walls from collenchyma. *Plant physiology*, **161**, 465-476.

Thomas, R. (1977) Wood: Structure and chemical composition: ACS Publications.

Timell, T. (1967) Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology*, **1**, 45-70.

Trache, D., Hussin, M.H., Haafiz, M.M. and Thakur, V.K. (2017) Recent progress in cellulose nanocrystals: sources and production. *Nanoscale*, **9**, 1763-1786.

Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigoriev, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S. and Salamov, A. (2006) The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). *science*, **313**, 1596-1604.

Usov, I., Nyström, G., Adamcik, J., Handschin, S., Schütz, C., Fall, A., Bergström, L. and Mezzenga, R. (2015) Understanding nanocellulose chirality and structure–properties relationship at the single fibril level. *Nature communications*, **6**, 1-11.

Vain, T., Crowell, E.F., Timpano, H., Biot, E., Desprez, T., Mansoori, N., Trindade, L.M., Pagant, S., Robert, S., Hofte, H., Gonneau, M. and Vernhettes, S. (2014) The Cellulase KORRIGAN Is Part of the Cellulose Synthase Complex. *Plant Physiol*, **165**, 1521-1532.

Van Acker, R., Leplé, J.-C., Aerts, D., Storme, V., Goeminne, G., Ivens, B., Légée, F., Lapierre, C., Piens, K. and Van Montagu, M.C. (2014) Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences*, **111**, 845-850.

Van Acker, R., Vanholme, R., Storme, V., Mortimer, J.C., Dupree, P. and Boerjan, W. (2013) Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in Arabidopsis thaliana. *Biotechnology for biofuels*, **6**, 1-17.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J. and Boerjan, W. (2010) Lignin biosynthesis and structure. *Plant physiology*, **153**, 895-905.

Voisin, H., Bergström, L., Liu, P. and Mathew, A.P. (2017) Nanocellulose-Based Materials for Water Purification. *Nanomaterials*, **7**, 57.

von Nägeli, C.W., Schwendener, S. and Leitgeb, H. (1858) *Beiträge zur wissenschaftlichen Botanik*: W. Engelmann.

Wang, Z., Zhu, J., Zalesny Jr, R.S. and Chen, K. (2012) Ethanol production from poplar wood through enzymatic saccharification and fermentation by dilute acid and SPORL pretreatments. *Fuel*, **95**, 606-614.

Wardrop, A. and Preston, R. (1947) Organisation of the cell walls of tracheids and wood fibres. *Nature*, **160**, 911-913.

Wickholm, K., Larsson, P.T. and Iversen, T. (1998) Assignment of non-crystalline forms in cellulose I by CP/MAS 13 C NMR spectroscopy. *Carbohydrate Research*, **312**, 123-129.

Wilkie, J. (1961) Carl Nägeli and the fine structure of living matter. *Nature*, **190**, 1145-1150.

Xu, P., Liu, H., Donaldson, L.A. and Zhang, Y. (2011) Mechanical performance and cellulose microfibrils in wood with high S2 microfibril angles. *Journal of Materials Science*, **46**, 534-540.

Ye, Z.-H. and Zhong, R. (2015) Molecular control of wood formation in trees. *Journal of experimental botany*, **66**, 4119-4131.

Youtie, J., Shapira, P. and Porter, A.L. (2008) Nanotechnology publications and citations by leading countries and blocs. *Journal of Nanoparticle Research*, **10**, 981-986.

Yu, H., Qin, Z., Liang, B., Liu, N., Zhou, Z. and Chen, L. (2013) Facile extraction of thermally stable cellulose nanocrystals with a high yield of 93% through hydrochloric acid hydrolysis under hydrothermal conditions. *Journal of Materials Chemistry A*, **1**, 3938-3944.

Zamil, M. and Geitmann, A. (2017) The middle lamella—more than a glue. *Physical Biology*, **14**, 015004.

Zhang, J., Nieminen, K., Serra, J.A.A. and Helariutta, Y. (2014) The formation of wood and its control. *Current opinion in plant biology*, **17**, 56-63.

Zhang, X., Dominguez, P.G., Kumar, M., Bygdell, J., Miroshnichenko, S., Sundberg, B., Wingsle, G. and Niittylä, T. (2018) Cellulose synthase stoichiometry in aspen differs from Arabidopsis and Norway spruce. *Plant physiology*, **177**, 1096-1107.

Zhong, R., Cui, D. and Ye, Z.H. (2019) Secondary cell wall biosynthesis. *New Phytologist*, **221**, 1703-1723.

Zhu, H., Jiang, S., Chen, H. and Roco, M.C. (2017) International perspective on nanotechnology papers, patents, and NSF awards (2000–2016). *Journal of Nanoparticle Research*, **19**, 1-11.

Zhu, H., Zhu, S., Jia, Z., Parvinian, S., Li, Y., Vaaland, O., Hu, L. and Li, T. (2015) Anomalous scaling law of strength and toughness of cellulose nanopaper. *Proceedings of the National Academy of Sciences*, **112**, 8971-8976.

Popular science summary

Cellulose is composed of long linear chains of glucose units which bundle together into fibrils with exceptional chemical and mechanical properties. Cellulose is the most abundant biopolymer on earth and the main component of wood. Wood has been used by human societies for thousands of years in construction and as energy source, and more recently in manufacturing of paper and packaging. The importance of wood is predicted to increase in the future as the demand for renewable materials, chemicals and fuels increases. Nanocellulose is a renewable and environmentally safe nanomaterial which can be derived from wood. The nanocrystals and nanofibrils of cellulose are 1000 times thinner than human hair and can be used in new innovative biobased nanomaterials including novel composites, absorbents or membranes, electronic devices and a spectrum of new medical applications. This PhD project combined wood biology and material science with an aim to improve nanocellulose isolation from wood. Currently, the use of modern tree breeding tools to improve the suitability and efficiency of wood for nanocellulose production is unexplored. This project shed light on underlying genetic factors controlling cellulose biosynthesis, cellulose microfibril dimensions and crystallinity as well as factors that facilitate nanocellulose extractability from wood. The results provide new insights and possibilities for future tree breeding to facilitate nanocellulose production from wood.

Populärvetenskaplig sammanfattning

Cellulosa är sammansatt av långa linjära kedjor av glukosenheter som binds ihop till fibriller med exceptionella kemiska och mekaniska egenskaper. Cellulosa är den biopolymer som det finns mest av på jorden och utgör huvudkomponenten i trä. Trä har använts av mänskligheten i tusentals år som byggmaterial, energikälla och mer nyligen i framställningen av papper och förpackningar. Betydelsen av trä förutspås i framtiden öka i takt med efterfrågan av förnybara material, kemikalier och bränsle. Nanocellulosa är ett förnybart och miljövänligt nanomaterial som kan utvinnas ur trä. Nanokristallerna och nanofibrillerna av cellulosa är 1000 gånger tunnare än ett mänskligt hårstrå och kan användas till nya innovativa biobaserade nanomaterial så som nya kompositmaterial, absorptionsmedel eller membran, elektroniska apparater och ett spektrum av medicinska applikationer. Det här doktorandprojektet kombinerade träbiologi och materialvetenskap med syfte att förbättra nanocellulosaisolering från trä. I nuläget är användningen av moderna träförädlingsverktyg för att förbättra lämpligheten och effektiviteten av trä för produktionen av nanocellulosa outforskad. Det här projektet kastar ljus över underliggande genetiska faktorer som styr syntesen av cellulosa, cellulosamikrofibriller, dess dimensioner och kristallinitet, samt faktorer som underlättar extraktionen av nanocellulosa från trä. Avhandlings resultat bidrar med insikter och nya möjligheter för träförädling i framtiden för att underlätta produktionen av nanocellulosa från trä.

Acknowledgements

Finally, I will close this chapter and leave the field open to someone else. But before I would like to thank many people that were part of this journey and contributed to this thesis.

First of all, I would like to thank my supervisor **Dr. Totte Niittylä**, for giving me the opportunity to do my PhD in your group and for this particular research project. I´m very grateful for your support and guidance during the years but also your trust and the freedom you gave me to decide and work independently.

Thank you very much, **Professor Kristiina Oksman** and **Simon Jonasson** from Luleå University of Technology (LTU), for enabling this interdisciplinary collaboration and for giving me insights in the world of material science and nanocellulose. I´m grateful for being part of this project.

A special thanks goes to all, the former and current group members making it a pleasure and fun to be part of this group.
Sonja, thank you for all the, lab and life related, discussions, company and friendship.
Junko, thank you for all your help and support in the cell wall lab and letting me help you with teaching, which was educational and always a lot of fun. And of course, for supplying us with sushi lunch every other week.
Wei, thank you for all your help and expertise in the lab during these years and answers to almost all my questions especially in the beginning.
Loïc, **Pieter, Romain, Sascha and Tayebeh**, thank you all for your help, advices and discussions during my time as a PhD student.

I also would like to thank **Prof. Ewa Mellerowicz**, **Prof. Stépahnie Robert** and **Dr. Ola Sundman** from my advisory group. Especially **Ola**, I have to thank for all your countless hours of help and support with the size-exclusion measurements.

Thanks to **Dr. Markus Rüggeberg**, for hosting me at ETH Zürich and helping with the measurements of the wood mechanical properties.

Thanks to **Swetree Technologies** for providing aspen samples as a raw material for our nanocellulose isolations.

I would like to thank my opponent **Prof. Ingo Burgert** and members of the evaluation committee, **Prof. Catherine Bellini**, **Prof. Francisco Javier Vilaplana Domingo** and **Prof. Silvia Larsson** for accepting this task and evaluating my thesis.

This whole work would definitely not have been possible without all the people who took care of my trees and transformations! A big thank you to the greenhouse team **Anna Brännström**, **Anna Forsgren**, **Åsa Gavelin**, **Ann Sehlstedt** and **Jennie Lönnebrink** and the poplar transgenic facility team especially **Iftikhar Ahmad**, **Veronica Bourquin**, **Verena Fleig**, and **Sonia Olmedo Díaz**.

I also want to thank all those people who keep UPSC running. Especially **Anne Honsel** for all your work with organizing and communicating, **Barbro Nylen** and **Inga-Lis Johansson** for the administrative work and help with my 1000 questions and **Simon Birve** for support with technical issues due to my lack of knowledge.

Many thanks to **all the people from UPSC** for your help, advice, kindness and open mindedness making UPSC such a nice and pleasant place to work!

I also need to say a big thank you to **Daria Chrobok** for illustrating my thesis front cover.

I want to thank **all my friends**, the old and new ones, for making my life outside work more social and enjoyable. Especially **Jenny**, who I am very grateful that I have met during our Biology Bachelor and being a dear friend since then.

Thank you to **my family** for letting me move so far away from home. All of you, I miss a lot!
Danke für eure Geduld und Akzeptanz mich, allem in allen, ganze 24 Jahre lang zur “Schule” gehen zu lassen! Jetzt wisst ihr jedenfalls auch was ich die letzten 4,5 Jahre so gemacht habe. Leider nichts erfunden, nichts patentiert aber immerhin ein wenig zur Grundlagenforschung beigetrategn, auch wenn es im grossen Zussamenhang nur ein kleiner Schritt war.

Finally, I want to express my deepest gratitude to **Jacob** for all your love and support since I started my PhD and **Ylvi** for inspiring me every day to keep on trying and that giving up is not an option. Jag älskar er!

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2021:73

Wood is one of nature's most versatile and sustainable raw materials which can be used to produce novel products such as nanocellulose. This thesis work combines wood biology and material science to investigate wood formation and wood chemistry in relation to the production and quality of nanocellulose.

Anne Bünder received her graduate education at the Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, Sweden. She completed her Master in Plant Science at the University of Bonn, Germany.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension as well as environmental monitoring and assessment are used to achieve this goal.

Online publication of thesis summary: http://epsilon.slu.se/eindex.html

ISSN 1652-6880
ISBN (print version) 978-91-7760-823-3
ISBN (electronic version) 978-91-7760-824-0