Sucrose Cleavage Pathways in Aspen Wood

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Cover: A model of sucrose hydrolysis and UDP-Glucose formation for cellulose biosynthesis in hybrid aspen wood.
(photo: *PhD* Kemal Avican, kavican@gmail.com)

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Abstract

Cellulose is the main component of wood and one of the most important renewable raw materials. In several tree species including *Populus* species, carbon for cellulose biosynthesis is derived from the disaccharide sucrose. This thesis describes experimental work on the mechanism of sucrose cleavage in developing wood and subsequent production of UDP-glucose (UDP-Glc) for cellulose biosynthesis.

Sucrose synthase (SUS) has been proposed previously to interact directly with cellulose synthase complexes (CSC) and specifically supply UDP-Glc for cellulose biosynthesis. To investigate the role of SUS in wood biosynthesis, transgenic lines of hybrid aspen (*Populus tremula* L. *x tremuloides* Michx.) with strongly reduced soluble SUS activity in developing wood were characterized. The reduction of soluble SUS activity to few percentage of wild type increased soluble sugar content but decreased wood density and consequently reduced the lignin, hemicellulose and cellulose content per volume of wood. The results demonstrate that SUS has an important role in carbon flux from sucrose to all wood polymers but has no specific role in supplying UDP-Glc to cellulose synthesis machinery.

I also investigated the role of cytosolic neutral/alkaline invertases (cNINs) during cellulose biosynthesis in hybrid aspen by analysing transgenic lines where NIN activity was decreased during secondary cell wall formation. The decrease in NINs activity caused a reduction in UDP-Glc and consequently reduced crystalline cellulose content but increased amorphous cellulose in cellulose microfibrils of wood. The results in this study demonstrated that cNIN activity is a major rate-controlling step in the cellulose biosynthesis.

There is a lack of global analytical methods to measure sugar phosphates linked to cell wall polymer biosynthesis. To address this problem, I worked with the UPSC metabolomics facility to develop a robust method based on chloroform/methanol extraction, two-step derivatization and detection using reverse phase liquid chromatography-mass spectrometry (RP-LC-MS) without adding ion-pairing reagent. The method could quantitatively identify 18 sugar phosphates including UDP-Glc and structural isomers in *Populus* leaf and wood extracts. The method can now be used to gain deeper understanding into wood metabolism and cell wall biosynthesis.

Keywords: Cellulose, sucrose synthase, invertase, cellulose synthase complex, UDP-glucose, UHPLC-MS/MS.

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Dedication

To my beloved Hazal and my family

The true guide in life is science.
Mustafa Kemal Ataturk

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 Lorenz Gerber, Bo Zhang, Melissa Roach, **Umut Rende**, Andras Gorzsas, Manoj Kumar, Ingo Burgert, Totte Niittylä and Bjorn Sundberg (2014). Deficient sucrose synthase activity in developing wood does not specifically affect cellulose biosynthesis, but causes an overall decrease in cell wall polymers. *New Phytologist*, 203:1220-1230.

II **Umut Rende**, Wei Wang, Madhavi Latha Gandla, Leif J. Jönsson and Totte Niittylä (2016). Cellulose biosynthesis in wood relies on cytosolic invertase activity. *New Phytologist, in press.*

III **Umut Rende**, Totte Niittylä and Thomas Moritz (2016). Determination of sugar phosphates in plants using combined reversed phase chromatography and tandem mass spectrometry. *Manuscript*.

Papers I is reproduced with the permission of the publishers.

The contribution of Umut Rende to the papers included in this thesis was as follows:

I Planning, designing and performing SUS enzyme activity measurement experiments, and writing and formatting the manuscript

II Planning, performance of the work, analysis and summary of the results, writing and preparation of the manuscript

III Planning, performance of the work, analysis and summary of the results, writing and preparation of the manuscript

Abbreviations

3-PGA	3-phosphoglycerate
2-PGA	2-phosphoglyceric acid
Ara	arabinose
AEC	anion exchange chromatography
CesA	cellulose
CMF	cellulose microfibril
CSC	cellulose synthase complex
CSL	cellulose synthase like
CWIN	cell wall invertase
DHAP	di-hydroxy-acetone phosphate
ESI	electro-spray ionization
EST	expressed sequence tag
FRK	fructokinase
Fru	fructose
Fru-6-P or F6P	fructose-6-P
Fru-1,6-P or FBP	fructose-1,6-bisphosphate
Fru-1,6-Pase	fructose-1,6-bisphosphatase
Gal	galactose
Gal1P	galactose-1-P
GalA	galacturonic acid
GAP	3-phosphoglyceraldehyde
GDP	guanine-di-phosphate
GFP	green fluorescence protein
Glc	glucose
Glc-6-P or G6P	glucose-6-phosphate
Glc-1-P or G1P	glucose-1-P
GlcA	glucuronic acid
GT43B	glycosyl transferase 43B

HXK	hexokinase
INV	invertase
IPC	ion-pairing chromatography
RP-LC-MS	reverse phase-liquid chromatography- mass spectrometry
Man	mannose
Man-6-P or Man6P	mannose-6-phosphate
MRM	multiple reaction monitoring
mtHXK	mitochondrial hexokinase
NDP	nucleotide-di-phosphate
NIN	neutral/alkaline invertase
cNIN	cytosolic neutral/alkaline invertase
PGI	phospho-gluco-isomerase
PGM	phospho-gluco-mutase
QqQ	triple quadrupole
R5P	ribose-5-P
Ru5BP	ribulose-1,5-PP
Ru5P	ribulose-5-P
Rubisco	ribulose-1,5 bisphosphate carboxylase-oxyganase
Sedu7P	seduheptulose-7-P
SPS	sucrose phosphate synthase
Suc	sucrose
Suc-6-P or S6P	sucrose-6-P
Sugar-P	sugar phosphate
SUS	sucrose synthase
Tre-6-P or T6P	trehalose-6-P
UDP	uridine-di-phosphate
UDP-Glc or UDP-G	UDP-glucose
UGD	UDP-glucose dehydrogenease
UGE	UDP-glucose-4-epimerase
UGPase	UDP-glucose pyrophosphorylase
UHPLC	ultra-high-performance liquid chromatography
UXS	UDP-xylose synthase
VIN	vacuolar invertase
Xyl	xylose
X5P	xylulose-5-P
YFP	yellow fluorescence protein

1 Introduction

1.1 Wood and wood formation

An estimated 15 % of the total atmospheric carbon passes through land plants every year, creating the largest annual carbon flux on the planet (Solomon *et al*., 2007). From this carbon flux, approximately 45% of carbon is stored in forest plants. Majority of this carbon accumulation occurs in non-photosynthetic plant cells. Wood as a non-photosynthetic tissue is one of the most abundant biomass on earth and is counted as the main carbon storage in terrestrial environment (Bonan, 2008).

For centuries, humans have used wood as a raw material for many purposes; construction, tools, as an energy source and more recently in pulp and paper industry. Nowadays wood is of significant interest as a sustainable raw material for new wood derived materials such as bioplastic, textile, vanillin or soap; and renewable fuels (Nieminen *et al.*, 2012; Plomion *et al.*, 2001)

Wood provides mechanical support necessary to hold the plant upright as well as the structure for the transportation of water and minerals from root to other parts of the plant. Wood is generated through the activity of the vascular cambium which contains vascular stem cells (Dejardin *et al.*, 2010). Wood formation is commonly divided into four developmental steps: cell division, cell expansion, secondary cell wall deposition and maturation (Fig. 1) (Mellerowicz *et al.*, 2001). Angiosperm wood contains mainly three different types of cells in wood: ray cells, vessels and fibres. These cells possess varied structural and functional duties. Ray cells are living parenchyma cells and provide a radial transport route between phloem and wood. Vessels or vessel

elements are specialized water conducting cells. They are dead hollow tube like cells and form secondary cell wall to strengthen the cell wall. Fibres are long fusiform cells with typically thicker secondary cell walls compared to vessel cells and provide mechanical support to tree (Plomion *et al.*, 2001).

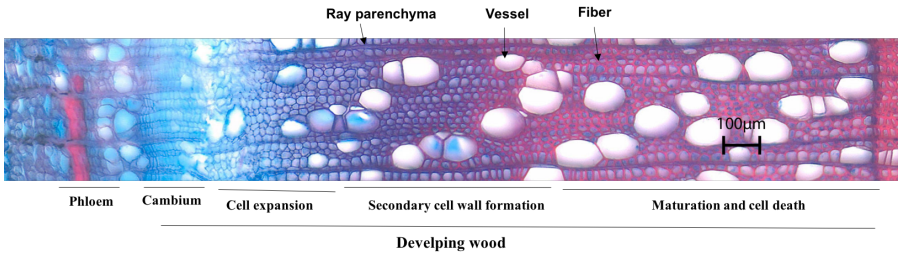

Fig. 1 Overview of the wood developmental stages (cambial division, cell expansion, secondary cell wall formation, cell death and maturation) and wood cell types (Ray parenchyma, vessel and fiber cells) (The image was kindly donated by UPSC Bioinformatics Facility)

Majority of the woody biomass resides in the secondary cell walls of wood fibers. The main components of secondary cell walls are cellulose, hemicellulose and lignin. Deposition of these components relies on imported carbon into the developing wood fibers. In most plants, carbon source for secondary cell wall formation is sucrose which is exported from photosynthetic tissues, then transported through phloem and finally incorporated into developing wood (Turgeon, 1996).

In the following sections, I will introduce how sucrose is formed in the photosynthetic source tissues and transported to developing wood cells and then I will focus on sucrose hydrolysis and cell wall formation.

1.2 Carbon assimilation, sucrose formation and transport

Carbon is fixed by photosynthesis through a sequence of enzymatic reactions where light and CO_2 are used to synthesize sugars in chloroplasts of photosynthetic cells (Calvin & Benson, 1949). The non-photosynthetic tissues rely on the photosynthesis derived sugars for energy and biosynthesis. In several tree species, including *Populus* sp., the assimilated carbon from photosynthesis is delivered in the form of sucrose (Suc) to developing wood (Turgeon, 1996). Suc is a non-reducing disaccharide composed of glucose (Glc) and fructose (Fru) that are linked by glycosidic linkage between C_1 of Glc subunit and C_2 of Fru subunit (Fig. 2).

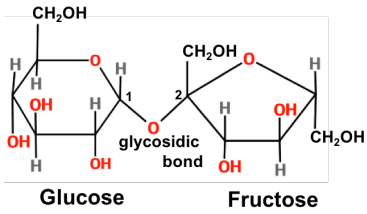

Fig. 2 The structure of sucrose which consists of one molecule of glucose and one molecule of fructose linked by an α-1,2 glycosidic linkage.

Precursors of Suc biosynthesis are formed through the Calvin cycle in chloroplast. Ribulose-1,5 bisphosphate carboxylase-oxygenase (Rubisco) carboxylates ribulose-1,5 bisphosphate to generate two molecules of 3-phosphoglycerate (3-PGA) (Miziorko & Lorimer, 1983). 3-PGA is then reduced to triose phosphates (TPs) and transported to cytosol through the TP translocator (Weber *et al.*, 2005). TPs are then converted to fructose 1,6 bisphosphate (Fru-1,6-P) by Fru-1,6-P aldolase. Through enzymatic reactions by Fru-1,6-Pase, hexose phosphate isomerase and phosphoglucomutase (PGI) respectively, Fru-1,6-P is converted to glucose 1-P (Glc-1-P) in the cytosol. Following this, UDP-glucose (UDP-Glc) is formed from Glc-1-P by UDP-Glc pyrophosporylase (UGPase). Suc-6-P synthase (SPS) produces Suc-P using one molecule of both UDP-Glc and Fru-6-P. Finally, Suc is formed by sucrose phosphate phosphatase which removes the phosphate from Suc-P.

In most investigated tree species, sucrose formed in the source tissue, which are carbohydrate exporters, is loaded into phloem passively (Rennie & Turgeon, 2009). The strongest evidence for *Populus* as a passive phloem loader was provided by Zhang *et al.,* (2014). In this study, the cell wall targeted yeast invertase, which cleaves apoplasmic sucrose to glucose and fructose, was expressed under 35S promoter or the minor vein specific *GALACTINOL SYNTHASE* promoter in grey poplar (*Populus tremula x alba*) and alfalfa (*Medicago sativa*), respectively. The phloem loading was impaired in the apoplasmic loader alfalfa, whereas no loading effect was observed in grey poplar. This indicated that sucrose was loaded to phloem through symplasmic transport route in *Populus* (Zhang *et al.*, 2014).

After transport through phloem, Suc is unloaded in sink tissues which are carbohydrate importers such as wood. In stem phloem, sucrose is unloaded from sieve tubes to ray cells and the transport continues through ray cells symplasmically. However, the investigation of sucrose transport from ray cells to developing wood in *Populus* using symplasmic fluorescent tracers did not

reveal any connections between rays and developing vessels or fibers (Sokolowska & Zagorska-Marek, 2012). It was suggested that sucrose is first actively exported from ray cells and then is imported into developing fibres and vessels. This was supported by Mahboubi *et al.,* (2013) where the reduction of a sucrose transporter expression by RNAi in developing wood during secondary cell wall formation resulted in decreased carbon allocation into secondary cell walls. It was concluded that active sucrose import is required to maintain secondary cell wall biosynthesis in developing wood (Mahboubi *et al.*, 2013).

After sucrose has been actively imported into wood cells, it is hydrolysed in order to join the sugar metabolism pathways. In the next section, I elucidate sucrose hydrolysis mechanisms in wood cells.

1.3 Sucrose hydrolysis mechanisms in wood

According to the current understanding, Suc in plants can be hydrolysed either by sucrose synthase (SUS) or invertase (INV) activity to supply energy and carbon to the cellular metabolism.

1.3.1 Sucrose Synthase (SUS):

SUS belongs to the glycosyltransferase family of enzymes and catalyses reversible cleavage of Suc. The reaction favours Suc degradation to form Fru and UDP-Glc in the presence of UDP at neutral pH in sink tissues (Fig. 3) (Geigenberger & Stitt, 1993; Delmer, 1972).

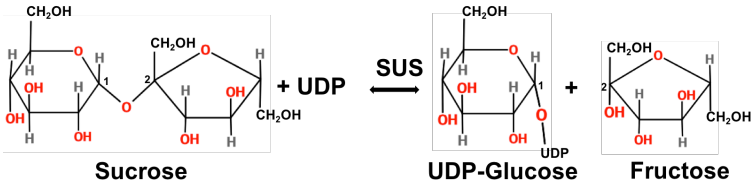

Fig. 3 Illustration of the reversible reaction of SUS.

Isoforms of *SUS* are expressed ubiquitously in plant tissues, and the SUS proteins have been localized to different compartments of a plant cell. The immuno-localization of SUS using an anti-SUS antibody suggested that SUS was associated with the membrane fraction in cotton (*Gossypium hirsutum*) fibers (Amor *et al.*, 1995) and a specific cotton SUS (SusC) was localized at the cell wall of cotton fibers (Brill *et al.*, 2011). The blotting of different cell

fractions from root tips and shoot using a maize (*Zea Mays*) anti-SUS antibody showed the localization of SUS in cytosol, mitochondria and nuclei (Subbaiah *et al.*, 2006). In another example, co-immuno-detection of Arabidopsis SUS2 and E37 which is a polypeptide of the inner plastid envelope showed SUS association with plastids of *Arabidopsis thaliana* embryo, endosperm and seed coat (Nunez *et al.*, 2008). In addition, immuno-localization studies in which a maize anti-SUS antibody was used, revealed presence of SUS in both purified tonoplast vesicles of red-beet (*Beta vulgaris*) hypocotyls (Etxeberria & Gonzalez, 2003) and Golgi membrane enriched fractions from maize coleoptile or soy bean (*Glycine max*) hypocotyl (Buckeridge *et al.*, 1999).

In summary, localization studies in different species so far have revealed the association of SUS proteins with plasma membrane (Amor *et al.*, 1995), cell wall (Brill *et al.*, 2011), mitochondria, cytosol, nuclei (Subbaiah *et al.*, 2006), plastid (Nunez *et al.*, 2008), tonoplast (Etxeberria & Gonzalez, 2003) and Golgi (Buckeridge *et al.*, 1999) indicating to diverse functions of SUS proteins.

The functional role of SUSs has been investigated in several species using genetic tools. Antisense inhibition of *SUS* in potato tubers reduced SUS activity in tubers and resulted in reduced starch, but increased sucrose, glucose and fructose content (Zrenner *et al.*, 1995). Consistent with this observation, reduced SUS activity in the double mutant of maize developing endosperm SUSs, *shs1* and *sus1*, showed a reduction in starch content (Chourey *et al.*, 1998). These studies suggested that sucrose synthase provides substrates for starch synthesis by breaking sucrose down. In tomato (*Lycopersicon chmielewskii*), it was shown that SUS activity was high during the fruit growth and almost no activity was detected in the mature tomato fruit. Since SUS activity correlated with fruit size, it was suggested that SUS in developing tomato fruit increases the fruit sink strength (Sun *et al.*, 1992). In a study of wheat (*Triticum aestivum*) roots, SUS activity was below detection level under well aerated conditions (Albrecht & Mustroph, 2003). However, SUS activity was increased in root meristem and lateral root under hypoxia where oxygen level was very low. Increased SUS activity under hypoxia correlated with an increase in soluble carbohydrate content and thickening of cell walls by increasing both cellulose and callose content. This study indicated that SUS has a role in UDP-Glc formation for the synthesis and thickening of the cell walls under hypoxia (Albrecht & Mustroph, 2003). The role of SUS in callose formation was supported by Barratt *et al.*, (2009). In Arabidopsis, two SUS isoforms out of six, which are SUS5 and SUS6, were shown to be localized in hypocotyl phloem by SUS5 and SUS6 specific antibodies. The double knock-

out *Arabidopsis* mutant of *sus5/sus6* caused collapsing sieve plates. In addition, immunogold labeling with anti-callose antiserum followed by transmission electron microscopy showed thinner callose layer in the mutant. It was concluded that SUS5 and SUS6 provide UDP-Glc for callose formation on phloem sieve plates (Barratt *et al.*, 2009). SUSs were also suggested to have role in cellulose biosynthesis where immuno-localization of SUS in cotton fibers showed plasma membrane association of SUS (Amor *et al.*, 1995). In this study, isolated cotton fibers were supplied with ^{14}C labeled Suc and the analysis resulted in production of both labelled cellulose and callose. Hence, SUSs activity was correlated with biosynthesis of glucan polymers at the plasma membrane.

It can be concluded that individual SUS isoforms are involved in the production of substrates which are used in starch, cellulose and callose biosynthesis. They are important for plant tissue and organ development including potato tuber (Zrenner *et al.*, 1995), wheat root (Albrecht & Mustroph, 2003), maize seed (Chourey *et al.*, 1998), tomato fruit (Sun *et al.*, 1992), cotton fibers (Amor *et al.*, 1995) and *Arabidopsis* root and stem (Barratt *et al.*, 2009).

A popular model of cellulose biosynthesis depicts a plasma membrane associated SUS in the supply of UDP-Glc directly to cellulose synthesis complex (CSC) (Fujii *et al.*, 2010; Haigler *et al.*, 2001; Amor *et al.*, 1995). This model is largely based on the observation that reduced SUS activity in transgenic cotton seed fibers caused reduced fiber initiation and elongation (Ruan *et al.*, 2003). It is worth to note that in these transgenic cotton lines, hexose sugar content was also reduced and this might affect sugar signalling and osmotic potential which could also lead to problems in fiber initiation and elongation. A direct association of CSC and SUS in *Populus deltoides x canadensis* developing wood was also suggested by Song *et al.*, (2010). In this study, SUS protein identified using proteomics analysis with antibody directed immuno-precipitation of CSCs. However, it was unclear whether the SUS protein, which was identified in immuno-precipitation of CSCs, was detected due to cytosolic contamination during the isolation steps. Moreover, overexpression of cotton *SUS* under *35S* and *4CL* promoter in *Populus alba x grandidentata* increased SUS activity by 2.5-fold and resulted in 2-6% increase crystalline cellulose (Coleman *et al.*, 2009). The effect of the increase in SUS activity was not limited to cellulose biosynthesis, but also affected hemicellulose content where mannose content was also increased and

arabinose content decreased (Coleman *et al.*, 2009). As a result, the support for specific involvement of SUS in cellulose biosynthesis is still unclear.

A serious challenge of the SUS-CSC model came with the observation that a quadruple knock-out (*sus1sus2sus3sus4)* mutant of *Arabidopsis* did not show any defects in growth or cell wall polymer biosynthesis. The remaining *SUS5* and *SUS6,* as explained above in this section, were detected only in phloem sieve elements and the sus5/sus6 double knock out lines showed thinner callose layer in the pores of sieve element plates. Hence, it was suggested that SUS5 and SUS6 had a role in callose formation, but alternative pathways could also supply UDP-Glc to cellulose biosynthesis (Barratt *et al.*, 2009). In the same study, in addition to quadruple *SUS* mutant, double knock-out of two neutral/alkaline invertases (NINs) which are highly expressed in the root of *Arabidopsis* were analysed. The *NIN*, *cinv1/cinv2,* mutant plants were much smaller compared to wild type (WT) and had reduced root growth with a tendency of root cells to collapse. It was suggested that this abnormality was due to a defect in cell wall biosynthesis, but the authors did not analyse any cell wall component in these mutant lines. Many of the conclusions of this study were subsequently challenged by measuring SUS activity in the quadruple mutant using optimized SUS assay conditions (Baroja-Fernandez *et al.*, 2012). In the study, it was claimed that the remaining SUS5 and SUS6 in *Arabidopsis* quadruple *sus1sus2sus3sus4* mutant had sufficient SUS activity to compensate the lack of SUS activity. Nonetheless, in this study, there is no indication of cell wall analysis in mutant lines and no debate on the role of SUS5 and SUS6 in callose synthesis. Although the activity measurement may not have been reliable in Barratt *et al.*, (2009), they showed convincingly the localization of SUS5 and SUS6 and the defect in callose formation in the stem. Moreover, Baroja-Fernandez *et al.*, (2012) did not provide an explanation how phloem localized SUS could compensate cell wall biosynthesis throughout the plant.

Consequently, the possible function of SUS in cellulose and cell wall biosynthesis remained unclear.

1.3.2 Invertases (INVs)

INVs provide an alternative sucrose cleavage method to SUS. As mentioned above and discussed in more detail here, INVs have recently emerged as a crucial enzyme for plant growth and development.

INV was first isolated from yeast and identified by Berthelot in 1860 and its chemical properties were first examined by Neumann & Lampen, (1967). INVs catalyse the irreversible hydrolysis of sucrose into Glc and Fru (Fig. 4). INVs are classified as cell wall, vacuolar and neutral/alkaline invertases according to their pH optima and cellular locations (Winter & Huber, 2000; Sturm, 1999).

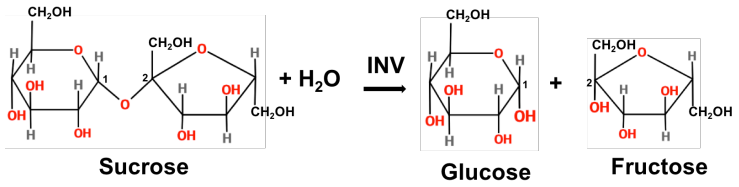

Fig. 4. Illustration of the irreversible reaction of INVs.

Acidic invertases: Cell wall invertases (CWIN) and vacuolar invertases

Acidic INVs are known as fructo-furanosidases which can also hydrolyse other fructofuranoses such as raffinose and stachyose, besides sucrose (Sturm, 1999). There are two types of acidic INVs: cell wall invertases (CWINs) and vacuolar invertase (VINs) (Bocock *et al.*, 2008). CWINs and VINs are localized in cell wall matrix or in vacuole, respectively. They have an optimum activity at acidic pH and are N-glycosylated (Tymowska-Lalanne & Kreis, 1998).

CWIN mutants have provided insights into the functional roles of this group of enzymes. In rice (*Oryza sativa*) grains, a loss of function mutant of the *CWIN* gene (*GIF1*) increased susceptibility to postharvest fungal pathogens (Sun *et al.*, 2014). Cell wall analysis of rice glume in *gif1* mutant showed thinner cell walls and reduced cytosolic glucose, fructose and sucrose content compared to WT. Overexpression of *GIF1* resulted in an increase in cytosolic sugars, but did not show any difference in cell wall thickness in leaf mesophyll cells compared to WT. Interestingly, after infection, overexpression lines showed enhanced fungal resistance and increased cell wall thickness. The thicker cell walls were shown to contain increased levels of xylose, cellulose and callose (Sun *et al.*, 2014). In addition, the expression of pathogen related genes was constitutively elevated after infection in overexpression lines. It was concluded that rice CWIN/GIF1 has a role in cell wall reinforcement and activates pathogen related defence by mediating sugar homeostasis under pathogen

attack. In tobacco (*Nicotiana tabacum*), pollen specific antisense repression of a *CWIN (NIN88*) generated male sterile plants, decreased pollen germination and pollen number, and had less number of seeds. It also reduced seed vessel development which resulted in loss of seed weight. This suggested that CWIN has an important role in male gametophyte development in tobacco (Hirsche *et al.*, 2009). In tomato (*Solanum lycopersicum*), RNAi silenced *CWIN* inhibitor (*INVINH1*) increased CWIN activity and following this Fru and Glc levels were also increased. The increase in CWIN activity resulted a delay in leaf senescence, and an increase in both seed and fruit size (Jin *et al.*, 2009). Although there is no discussion on whether the improvement in fruit development was a secondary effect from the delayed senescence, the increased activity of cell wall localized INV in tomato might be an indication for the primary effect of CWIN in fruit and seed development. The study in hybrid *Populus* (*Populus alba L x grandidentata Michx.*) showed that the expression of one of the two *CWIN*s (*PaxgINV2*) was detected in all tissues with elevated expression during actively growing tissues, whereas *PaxgINV1* had elevated expression during dormancy (Canam *et al.*, 2008). It was hypothesized that CWINs in *Populus* sp. are involved in phloem unloading and providing sugars in growing tissues. In summary, these studies indicated that CWINs have a role in reinforcement of cell wall under pathogen attack (Sun *et al.*, 2014), phloem unloading (Canam *et al.*, 2008), timing senescence and fruit, pollen and seed development (Jin *et al.*, 2009).

VINs have been shown to have a role in hexose accumulation, cell division and cell expansion. Comparison of two cotton genotypes, *Gossypium barbadense* (*Gb*) which has seed fibers longer than *Gossypium hirsutum* (*Gh*) showed that longer fibers had more VIN (*Gh*VIN1) activity and Fru, Glc and Suc concentrations were elevated (Wang *et al.*, 2010). In the same study, overexpression of *GhVIN1* and inhibition of *GhVIN1* in cotton fiber cells elevated or reduced fiber elongation, respectively. Hence, VINs activity correlated to cell expansion and osmotic regulation by regulating hexose and Suc concentrations in cotton fiber cells. In another study, quantitative trait loci (QTL) detected for root and hypocotyl length revealed a positive correlation between a *VIN* and root/hypocotyl elongation in *Arabidopsis*. The *VIN* gene (At1g12240) pinpointed by the QTL analysis for root length was shown to be involved in root cell expansion by subsequent mutant analysis. Null mutant *vin* plants had shorter roots and suggested that this VIN has an important role in cell division and cell elongation (Sergeeva *et al.*, 2006). In summary, VINs are important for osmotic regulation by mediating hexoses and sucrose

concentrations both in dividing and expanding cells (Wang *et al.*, 2010; Sergeeva *et al.*, 2006).

There are no published studies directly linking acidic INVs to cell wall polymer biosynthesis. It is worth to note that overexpression of the *CWIN* (*GIF1*) gene in rice leaf exhibited more hemicellulose, cellulose and callose content in the cell wall only during infection (Sun *et al.*, 2014). Moreover, increased CWIN activity by overexpression of a yeast *CWIN* in *Populus* affected neither growth rate nor sugar levels. However, mutant lines showed necrotic symptoms in leaves under high temperature condition and had more callose compared to WT (Zhang *et al.*, 2014). These results indicated that CWINs might be involved in cell wall polymer biosynthesis under stress conditions, but it is unclear whether this involvement is in cell wall signalling or in supplying sugars to polymer synthesis machinery.

Neutral/alkaline invertases (NINs):

NINs also catalyse the irreversible hydrolysis of sucrose to Glc and Fru. In contrast to acidic invertases, NINs have an optimum activity at neutral or alkaline pH (pH 6.8-8.5), catalyse only Suc hydrolysis and are not glycosylated. NINs are closely related to cyanobacterial INVs and are thought to have their evolutionary origin in the endosymbiotic ancestor of chloroplasts (Vargas *et al.*, 2003). NINs are also closely related to periplasmic INVs of aerobic bacteria and respiratory eukaryotes such as yeasts (Ji *et al.*, 2005).

NINs have been identified in several plant species. For example, *Populus* has 16 genes (Bocock *et al.*, 2008), *Arabidopsis* 9 genes (Vargas *et al.*, 2003), and rice 8 genes (Ji *et al.*, 2005). According to the current nomenclature, *NIN*s are classified into α and β clusters based on amino acid similarity and exon/intron structures. *NINs* in α cluster (*NIN 1-7*) were predicted to localize in cell compartments (Bocock *et al.*, 2008). *In vivo* studies using fluorescent protein fusion have confirmed that NINs belonging to α clade in rice and *Arabidopsis* were localized to mitochondria and chloroplast, respectively (Xiang *et al.*, 2011; Murayama & Handa, 2007). However, the members of β cluster (*NIN 8-16*) are predicted as cytosolic NINs (Bocock *et al.*, 2008). This prediction was also confirmed in both tobacco and *Arabidopsis* leaf protoplasts by tagging a β-clade NIN protein with a fluorescent protein (Liu *et al.*, 2015; Xiang *et al.*, 2011).

NINs are expressed in different organs and tissues implying they play important roles in various biological processes in plants. Mutant studies of organelle localized NINs revealed important functions in mitochondria and chloroplast. For example, in *Arabidopsis*, the knock-out mutant of a *NIN* (*At-A/N-InvA)* which was located in mitochondria had reduced shoot and leaf development, and had short roots compared to WT (Xiang *et al.*, 2011). The authors hypothesized that NINs might mediate the supply of Glc to mitochondria associated Hexokinase (mtHXK). Due to the link between mtHXK and reactive oxygen species homeostasis (Bolouri-Moghaddam *et al.*, 2010) the expression level of both mtHXK and ROS genes were analysed in *A/N Inv-A* mutants. The analysis showed an elevated expression of both mtHXK and ROS genes in mutant plants. In contrast, transient overexpression of *At-A/N-InvA* in *Arabidopsis* mesophyll protoplasts down regulated *APX2* promoter which is known to respond to oxidative stress. The authors proposed that mitochondrial NIN contributed to ROS homeostasis by supplying Glc to mtHXKs in mitochondria (Xiang *et al.*, 2011). Another *Arabidopsis* NIN, At-A/N-InvE was shown to localize to chloroplasts using green fluorescent protein (GFP) tag. A T-DNA mutant of *At-A/N-InvE* did not show any difference in Suc content, but had lower starch content. This NIN was hypothesized to have a function in regulating carbon portioning between the cytosol and the chloroplast starch by Suc hydrolysis in chloroplast (Vargas *et al.*, 2008). The localization of NINs in plastids is intriguing since it is unclear whether plastids contain any Suc. Although Gerrits *et al.*, (2001) suggested the presence of Suc in chloroplasts, it is still unknown how Suc would be transported into chloroplast.

Cytosolic NINs (cNINs) have a general role in plant growth. In *Lotus japonicus*, a mutation in *cNIN* (*LjINV1*) resulting in its activity being reduced, caused a reduction in root and shoot growth and had a deficiency in flowering (Welham *et al.*, 2009). Similar, a mutation in a *cNIN* (*Oscyt-inv1*) in rice resulted short root phenotype and defect in pollen fertility. This mutant had higher sucrose and lower hexoses in both root and leaf compared to WT and addition of exogenous Glc rescued the phenotype. This study led authors to conclude that cNINs have a critical role in root and pollen development in rice by supplying hexoses to the cell metabolism (Jia *et al.*, 2008). In wheat, it was reported that the knock-down mutation of a *cNIN* (*Ta-A/N-Inv1)*, accumulated H_2O_2 and increased occurrence of cell death during *Puccinia striiformis (Pst)* pathogen infection. This mutant had an increase in photosynthesis rate during infection and elevated transcript levels of chloroplast localized ROS associated genes. This study proposed that cNINs in wheat may regulate photosynthesis

by hexose accumulation to avoid cell death due to excessive ROS production during infection (Liu *et al.*, 2015). All these studies indicate a central and general role for cNINs in Suc cleavage in plants and the cNIN activity reduction in plants may cause growth phenotypes ranging from leaf, shoot and root growth defects to male sterility. However, it is not possible to conclude from current studies whether the cause of phenotypes was the primary effect or a secondary effect of reduction in cNIN activity.

A link between NINs and cell wall polymer biosynthesis has not been studied so far, but the lack of obvious phenotypes in *SUS* mutant plants indicated a dominant role of NINs over SUS in cell wall biosynthesis. Near absent SUS activity in transgenic alfalfa (*Medicago sativa*) stems had no effect on cell wall composition but caused an increase in NIN activity. It was speculated that NINs instead were involved in substrate supply for cellulose biosynthesis in alfalfa (Samac *et al.*, 2015). Moreover, the double knock-out mutant of *cNINs* (*cinv1cinv2*) in *Arabidopsis* resulted in dwarf plants and reduced root growth (Barratt *et al.*, 2009). Having no obvious phenotype in *Arabidopsis* quadruple *sus1sus2sus3sus4* mutant (Barratt *et al.*, 2009) and the SUSRNAi hybrid aspen lines in (Gerber *et al.*, 2014) (Paper I) led us to question whether INV pathway is contributing to cell wall, especially to cellulose biosynthesis in the developing wood of aspen.

1.4 Biosynthesis of cell wall components in developing wood

The carbohydrate polymers of wood cell walls are synthesized from nucleotide-diphosphate sugars (NDP-sugars), such as UDP-Glc, UDP-Gal or GDP-Man. NDP-sugars are activated forms of monosaccharides in that they contain a high energy bond and hence have ability to transfer this energy to form glycosidic bonds. NDP-sugars are in most cases formed from hexose phosphate sugars which are produced after Suc hydrolysis in wood cells (Fig. 5).

As described above, SUS can produce UDP-Glc and Fru (Delmer, 1972). Alternatively, INV catalyzes Suc and produces Glc and Fru (Neumann & Lampen, 1967). Glc enters the hexose-phosphate pool by HXK conversion to Glc-6-P. Fru is irreversibly phosphorylated either by fructokinases (FRK) (Medina & Sols, 1956) or hexokinases (HXK) (Berger et al., 1946) to form Fru-6-P. Fru-6-P is substrate for phospho-manno-isomerase (PMI) to form mannose-6-phosphate (Man-6-P) (Slein, 1950) or phospho-gluco-isomerase (PGI) (Tsuboi et al., 1958) to form Glc-6-P. Both Man-6-P and Glc-6-P are targeted by their mutase enzymes, phospho-manno-mutase (PMM) (Small &

Matheson, 1979) and phospho-gluco-mutase (PGM) (Joshi et al., 1964) to produce mannose-1-phosphate (Man-1-P) and glucose-1-phosphate (Glc-1-P), respectively. UDP-Glc pyrophosphorylase (UGPase) converts Glc-1-P to UDP-Glc (Kamogawa & Kurahashi, 1965) and GDP-Man pyrophosphorylase (GMP) produces GDP-Man (GDP-Man) (Peaud-Lenoel & Axelos, 1968). The described enzymes in Suc cleavage, hexose phosphate formation and interconversion are soluble in cytosol and the sugars in these pathways are produced in cytosol.

NDP-sugars which are used as precursors for cell wall biosynthesis are derived from UDP-Glc, except GDP-Man and GDP-Fucose. UDP-Glucoronic acid (UDP-GlcA), UDP-Galactose (UDP-Gal), and UDP-Rhamnose (UDP-Rha) are derived from UDP-Glc interconversion by UDP-Glc dehydrogenease (Sergeeva *et al.*, 2006; Maxwell *et al.*, 1956), UDP-Glc-4-epimerase (Maxwell & Derobichonszulmajster, 1960) and UDP-Glc 4,6 hydrolyase (RHM) (Kamsteeg *et al.*, 1978), respectively. In addition, UDP-GlcA can also be produced by *myo*-Inositol oxidation pathway (Fig. 5). *Myo*-Inositol is formed through the conversion of Glc-6-P to *myo*-Inositol-1-P and then to *myo*-Inositol by Inositol-1-P synthase ($InsP_1Sy$) (Eisenberg, 1967) and Inositolphosphate-phosphatase (Gee *et al.*, 1988), respectively. *Myo*-Inositol oxygenase (MIOX) converts *myo*-Inositol to GlcA (Charalampous, 1959) and following, GlcA is converted to GlcA-1-P by glucuronokinase (GK) (Neufeld *et al.*, 1959). GlcA-1-P is then converted to UDP-GlcA by UDP-GlcA pyrophosphorylase (Roberts, 1971) or UDP-Sugar pyrophosphorylase (USPase) (Kotake *et al.*, 2004).

UDP-GlcA is the precursor for UDP-Xylose (UDP-Xyl) (Ankel & Feingold, 1965), and UDP-Galacturonic acid (UDP-GalA) conversion of which is catalysed by their respective glycosyl transferases, UDP-Xyl synthase (UXS) and UDP-GlcA epimerase (UGlcAE). In addition, UDP-Arabinose (UDP-Ara) is formed from UDP-Xyl by UDP-Ara 4-epimerase (Lerouxel *et al.*, 2006; Feingold *et al.*, 1960). The enzymes responsible for NDP-sugar formation are mainly located in cytosol. However, 4-epimerases acting on UDP-GlcA/UDP-GalA and UDP-Xyl/UDP-Ara are located in Golgi cisternae (Barber *et al.*, 2006; Seifert, 2004; Burget *et al.*, 2003).

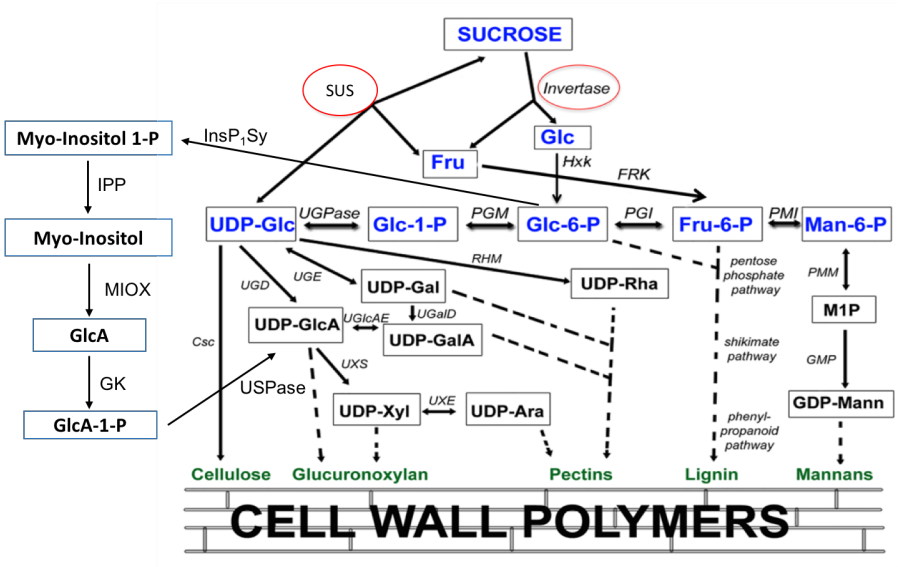

Fig. 5 Model of metabolic pathways from sucrose to cell wall polymers in aspen wood.

1.4.1 Cellulose Biosynthesis

Cellulose is the most abundant biopolymer on earth and the main component of plant cell walls (Delmer & Haigler, 2002). It has a role in determining the physical characteristics and strength of the cell wall and in providing mechanical support to the plant (Nishiyama, 2009). Cellulose is formed by individual β-1-4 glucan chains which can crystallize to form cellulose microfibrils (CMFs) through inter and intra hydrogen bonds and Van der Waals interactions (O'Sullivan, 1997). In plant cells, most of the cellulose forms CMFs which have some amorphous (non-crystalline) regions located at the surface of crystalline CMFs and are not arranged in a specific manner (Kondo *et al.*, 2001).

Cellulose is biosynthesized at the plasma membrane by cellulose synthase complexes (CSCs). Freeze fracture experiments on maize mesophyll parenchyma where the tissue cuts were stained with gold and rapidly frozen in liquid nitrogen followed by an electron microscopy analysis proposed that a hexameric rosette particles localized at the terminal part of CMFs on plasma membrane was responsible for cellulose synthases (Mueller & Brown, 1980). After, clear evidence of CSC's involvement in cellulose biosynthesis was shown in a radially swelling mutant in *Arabidopsis* (Arioli *et al.*, 1998). The mutation caused a single amino acid change in *Arabidopsis* cellulose synthase A (CesA) and caused deficiency in cellulose formation with a loss of rosette

structures in the plasma membrane. Further evidence of the connection between CesAs and CSC was shown in immuno-gold localization of cotton CesA protein in rosettes of freeze fractured membranes (Kimura *et al.*, 1999). According to latest models in plants, a CSC forms a hexagonal rosette and consists of 18 - 36 CesAs to produce 18-24 CMFs (Hill *et al.*, 2014; Newman *et al.*, 2013).

CesAs are known as glycosyl transferase proteins and utilize UDP-Glc as a substrate to produce glucan chains in order to form CMFs (Glaser, 1958). As explained in section 1.4, UDP-Glc can be supplied either by SUS or the NIN pathway in plant cells. SUS was suggested to have a direct association with CSCs and directly channels UDP-Glc to CesAs (Fujii *et al.*, 2010; Haigler *et al.*, 2001; Amor *et al.*, 1995). However, quadruple knock-out in four of six *Arabidopsis SUS*s did not have any defect in cellulose (Barratt *et al.*, 2009). In addition, double knock-out of *Arabidopsis UGPase*s showed a phenotype which was associated with cellulose decrease (Park *et al.*, 2010). In another study, inhibition of fructokinase (FRK) reduced cellulose level in hybrid aspen wood (Roach *et al.*, 2012). These studies indicated that SUS model where SUS directly supplies UDP-Glc to CSCs is unlikely. Hence, the carbon allocation to cellulose remained unclear.

1.4.2 Hemicellulose biosynthesis

Hemicelluloses are cross-linking glycans which are one of the major components of plant cell walls. They are heterogeneous polysaccharides that are formed by β-1,4 glycosidic bond at the backbone of the polymer. They bind both to cellulose and lignin by hydrogen-bonds and Van der Waals forces (Lerouxel *et al.*, 2006) or covalent bond, respectively (Carpita, 1996). In *Populus* wood, major types of hemicellulose polysaccharides are glucoronoxylan, glucomannan and xyloglucan. Glucoronoxylan is the most abundant hemicellulose in *Populus* wood that has an acetylated β-1,4 xylan backbone with α-1,2 glucoronic acid side-chains (Mellerowicz *et al.*, 2001). Second most abundant hemicellulose is glucomannan which has mixed β-1,4 glucose and β-1,4 mannan units at the backbone (Kim & Daniel, 2012). Xyloglucans are mostly found in primary cell walls of wood. It has β-1,4 glucan chain backbone and α-1,6 xylose side chains (Hoffman *et al.*, 2005).

Hemicelluloses are synthesized in Golgi by cellulose synthase like (CSL) proteins and glycosyltransferases (GTs), and then transported to the cell wall by vesicles. Both CSLs and GTs are Golgi membrane localized proteins. They

use NDP-sugars (section 1.4) as substrate and add monosugars to the reducing end of the backbone (Pauly *et al.*, 2013; Lerouxel *et al.*, 2006).

1.4.3 Lignin Biosynthesis

Lignin is a heteropolymer synthesized by laccases and peroxidases catalysing radical coupling of different monolignols which are aromatic alcohols. Lignin is mainly composed of three monolignols: sinapyl alcohol (S lignin), coniferyl alcohol (G lignin) and p-coumaryl (H lignin) (Boerjan *et al.*, 2003). Lignin polymer in angiosperm tree species consists mainly of S and G lignin subunits.

Carbon source for lignin biosynthesis is derived from Fru-6-P and Glc-6-P which are formed after sucrose hydrolysis in the plant cell. Both Fru-6-P and Glc-6-P are used as precursor for pentose phosphate pathway products of which are precursors of shikimate pathway in plastids. One of the end products of shikimate pathway is phenylalanine which enters the phenylpropanoid pathway to produce monolignols (Herrmann & Weaver, 1999).

1.5 Analysis of metabolites by LC-MS

The intermediates of Calvin cycle, sucrose and starch synthesis pathways in photosynthetic tissues, and cell wall polymer biosynthesis pathway in wood tissues consist mainly of sugar phosphates (sections 1.2 and 1.4). Hence, determination and quantification of sugar phosphates is important to investigate central carbon metabolism in plants. This requires a simple and robust extraction of metabolites and a detection method which should be sensitive, rapid and also handle different types of compounds such as polar, ionic or hydrophobic compounds.

There are three prevalent examples of metabolite extraction methods for plant materials. First one is chloroform-methanol extraction which is the most commonly used extraction method (Lunn *et al.*, 2006; Gullberg *et al.*, 2004). This extraction method is suitable to extract water soluble and organic soluble metabolites. Second one is hot ethanol extraction. It is suitable to extract polar and mildly non-polar metabolites (Bieleski, 1964). Although this method is easy to do, it requires ethanol extraction steps by heating ethanol. Some enzymes may remain active during heating steps and digest some metabolites. Third one is tri-chloro-acetic acid (TCA)-ether extraction method. This is suitable for acid stable and water soluble metabolites (Jelitto *et al.*, 1992).

To detect metabolites, the combined liquid chromatography-mass spectrometry (LC-MS) is an ideal method to identify and quantify compounds which have low molecular weight (Nordstrom *et al.*, 2004). MS can detect compounds which have an electrical charge (ions) based on mass to charge ratio (m/z). Depending on ionization technique, the molecular ions are fragmented into smaller ions. When very little fragmentation is occurring after ionization, isomeric compounds, such as Glc-6-P, Glc-1-P and Fru-6-P, show similar mass spectra and cannot be distinguished. To overcome this problem, MS is connected to a LC which separates molecules based on the interaction with mobile and stationary phases through a column (Huber & Oberacher, 2001). In summary, the separated metabolites in LC system must be first ionized before entering the MS. Following this, masses are separated in the mass analyser and then identified by a detector.

Different LC-MS techniques were used to profile sugar phosphates. For example, anion exchange chromatography connected to tandem mass spectrometry (AEC-MS/MS) was able to detect metabolites including Glc-6-P, Glc-1-P, Fru-6-P, Trehalose-6-P (Tre-6-P) and UDP-Glc. However, metabolites were not eluted completely in the column and triose phosphates were not detected (Arrivault *et al.*, 2009). In another study, it has been shown that using volatile alkylamine tributylamine as an ion pair compound (IPC-MS/MS) can help to separate organic acids and most of the phosphorylated molecules (Lou *et al.*, 2007), but this approach had some limitations. The major problem in IPC-MS/MS was that ion-pairing reagents interfered with the LC-MS/MS instrument and were difficult to wash out from the LC-system, and therefore the instrument might not be possible to use for other applications. In addition to that, it failed to detect Tre-6-P, Suc-6-P and PGA, and these molecules were quantified by enzymatic reactions (Arrivault *et al.*, 2009).

Reversed phase LC cannot be used for highly polar metabolites since they do not retain on a C18 column. This may cause ion suppression problems while using electrospray ionization due to large amount of unretained molecules in the columns competing for charges during ionization (Annesley, 2003). To overcome this problem, it was shown that including a derivatization step after extraction of polar molecules increased their hydrophobicity and improved chromatographic retention (Nordstrom *et al.*, 2004). In the same study, cytokinins and nucleotides were derivatized either by propionyl or benzoyl groups, but it was not shown how derivatization affects other polar molecules.

All taken together, the field is still missing a robust and easy detection of polar metabolites.

2 Objectives

My overall aim in this study was to define the sucrose metabolism pathway in secondary cell wall forming cells with a focus on UDP-Glc production for cellulose biosynthesis in hybrid aspen (*Populus tremula* L. *x tremuloides* Michx.) and develop a method to extract and quantify sugar phosphates and UDP-Glc.

Specific goals of my project were:

To investigate the role of soluble sucrose synthases (SUSs) in cellulose and wood biosynthesis in hybrid aspen (paper I)

To characterize cytosolic neutral/alkaline invertases (cNINs) and to investigate the role of cNINs in cellulose and wood formation in hybrid aspen (paper II)

To develop an LC-MS method to determine and quantify extracted sugar phosphates from plant materials (paper III)

3 Material and Methods

In this section, I give a description of the material and methods which are not found in the attached papers.

3.1 Model organism

In this study, hybrid aspen (*Populus tremula* L. *x tremuloides* Michx.) was used as the model tree species for the following reasons. Hybrid aspen can produce a large amount of woody biomass in a short period of time making it well suited for the study of wood formation. *Populus* sp. are phylogenetically relatively close to *Arabidopsis* allowing the transfer of knowledge from *Arabidopsis* studies to *Populus* (Jansson & Douglas, 2007). Moreover, *Populus* can be genetically modified by *Agrobacterium* mediated transformation which gives the possibility to create mutant lines with altered expression of targeted genes to investigate their biological function (Nilsson *et al.*, 1992). The genome of *Populus trichocarpa* was the first sequenced tree genome. The genome is 500Mbp and approximately four times larger than *Arabidopsis* genome. It consists of 19 chromosomes and 40000 gene models (Tuskan *et al.*, 2006); https://phytozome.jgi.doe.gov). In addition, availability of extensive gene expression data for *Populus* (http://popgenie.org) facilitates functional genetics studies.

3.2 Study approach to investigate *SUS*s and *INV*s

There are two types of genetic screens to study the function of a gene: reverse genetics and forward genetics. Forward genetics is a phenotype-centric approach which is used to find the genetic basis of a phenotype. In contrast to forward genetics, reverse genetics aims to find the phenotype which is the

result of an engineered specific gene (Alonso & Ecker, 2006). Availability of whole genome and mRNA sequence databases and improvements in gene cloning have made reverse genetics approach a faster, cheaper and easier way to test the hypothesis about specific gene functions.

Reverse genetics studies require first a selection of a candidate gene from the available databases based on its expression level in the tissue or the cellular/physiological process of interest. Second, mutant lines of the candidate genes are generated using one of the different methods. Some examples of these methods are insertional mutagenesis by T-DNA or transposon insertion, targeting induced local lesions in genomes (TILLING), RNA interference (RNAi), micro RNA (miRNA) (Alonso & Ecker, 2006) or CRISPR/Cas9 mediated gene silencing (Jinek *et al.*, 2012).

In this study, reverse genetics approach was used to investigate the function of *Populus tremula x tremuloides* SUSs and INVs in cellulose biosynthesis of wood cells. The candidate genes for *SUS*s, *SUS1* and *SUS2*, were selected based on their expression profile in developing wood using expressed sequence tag, sequencing and microarray data (Geisler-Lee *et al.*, 2006; Hertzberg *et al.*, 2001). The candidate gene for *INV*s, *cNIN12*, was selected based on its expression profile in developing wood which was obtained from microarray data (Hertzberg *et al.*, 2001). The selected genes were down regulated by using the RNAi mediated gene silencing approach (Hannon, 2012). *SUS1* and *SUS2* genes were targeted by *35S* promoter and *cNIN12* was targeted to secondary cell wall forming phase of developing wood cells by *GT43B* (*p-GLYCOSYLTRANSFERASE-43B*) promoter (Ratke *et al.*, 2015). The mutant lines with reduced transcript levels of candidate genes were selected for further characterization.

3.3 Analysis of metabolites by reverse phase LC-MS/MS

In this study, tandem mass spectrometry (MS/MS) was connected to reverse phase liquid chromatography (RP-LC) where the metabolites bind to a silica based C_{18} column by hydrophobic interactions in the presence of a hydrophilic solvent (for instance, water) and are eluted off by a more hydrophobic solvent like methanol or acetonitrile. After separation in LC, molecules were ionized using electrospray ionization (ESI) and were detected with MS/MS.

As it was mentioned in section 1.5, reversed phase liquid chromatography cannot be used for highly polar metabolites due to lack of retention on a C_{18}

column and may cause an ion suppression problem while using ESI (Jessome & Volmer, 2006). To overcome this problem and improve chromatographic retention of polar molecules, consecutive derivatizations were done to increase hydrophobicity. Samples were first derivatized by methoxylamine (CH_3ONH_2) which reacts with carbonyl groups to form an oxime derivative (-CH_3ON) (Fig. 6; paper III, Fig. 1) (Gullberg *et al.*, 2004). The derivatization was continued by applying methylimidazol and propionic acid anhydride to esterify hydroxyl groups by propionylation (Fig. 6; paper III, Fig. 1) (Nordstrom *et al.*, 2004). These derivatizations provided more hydrophobicity, less diversity in polarity of compounds and better ionization during ESI process.

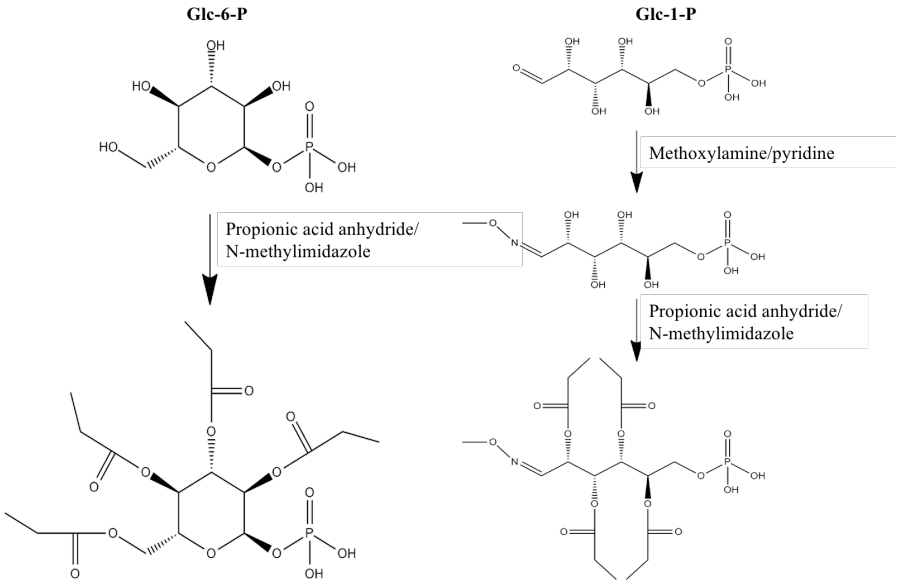

Fig. 6 Examples of derivatized molecules. This figure illustrates propionylation of Glc-6-P and derivatization of Glc-1-P by methoxyamine/pyridine and methylimidazol/propionic acid anhydride.

To identify analytes after ionization, Quadrupole-Time of Flight (QTOF) was used as a tandem mass analyser in paper III. A quadrupole (Q) is a mass analyser which has four cylindrical metal rods placed in parallel to form a cavity along the central axis of rods. The ions entering to the cavity are oscillated and masses are separated depending on both ion motion in the dynamic electric field and mass-to-charge ratio (m/z) of the ion (Glish & Vachet, 2003). TOF mass analyser measures the time of flight through a tunnel under low vacuum conditions for an ion which has a specific m/z ratio

(Weickhardt *et al.*, 1996). In QTOF, precursor ions, which are selected in the quadrupole, are fragmented in a collision cell and then fragments are measured in TOF. This MS/MS provides a high resolution and a high accuracy of mass measurement, hence it is suitable for identifying analytes.

The quantification of analytes in paper III was done by triple quadrupole (QqQ) tandem mass analyser in multiple reaction monitoring (MRM) mode. In QqQ, first and last quadrupoles are used as mass analysers and the middle quadrupole is used as a collision cell where ions are fragmented. In the first quadrupole precursor ions and in the second one product ions can be monitored. In this MS/MS, single reaction monitoring (SRM) mode is used to monitor one precursor-product ion. On the other hand, to monitor more than one precursor-product ion combinations, multiple reaction monitoring (MRM) is used (Glish & Vachet, 2003). MRM mode has high sensitivity and selectivity; hence it is well suited for quantification of analytes. However, MRM has low resolution, but a good chromatographic separation can compensate this disadvantage.

4 Results and Discussion

4.1 Deficient sucrose synthase activity in developing wood does not specifically affect cellulose biosynthesis, but causes an overall decrease in cell wall polymers (paper I)

The discovery of a plasma membrane associated SUS in developing wood led to a model of direct association between SUS and CesA machinery, where SUS supplies UDP-Glc directly to the cellulose synthase complex (CSC) for cellulose biosynthesis (Fujii *et al.*, 2010; Haigler *et al.*, 2001; Amor *et al.*, 1995). Since this depicted model was based on observations from SUS immunolocalization analyses, the role of SUS in cellulose biosynthesis was questioned by Barratt *et al.*, (2009) where *Arabidopsis sus1sus2sus3sus4* mutant showed no difference in plant growth and cellulose percentage in stems compared to WT. However, this study was challenged by the work of Baroja-Fernandez *et al.*, (2012), in which it was claimed that the remaining *SUS5* and *SUS6* in quadruple *sus* mutant were enough to compensate SUS activity. Therefore, evidence for the essential role of SUS in cellulose biosynthesis were still missing.

In paper I, to investigate the role of SUSs in cellulose and cell wall biosynthesis, the most abundant *SUS* transcripts, *SUS1* and *SUS2* (paper I; Fig. S1a, 1c), in developing wood were downregulated using RNAi under the *35S* promoter. Three *SUSRNAi* lines with reduced transcript abundance of both *SUS1* and *SUS2* to a small percentage of WT (Fig. 7a, 7b; paper I, Fig. 1a, 1b) were selected for further characterization. Consistently, the reduced *SUS1* and *SUS2* transcript levels resulted in a decrease in SUS activity to 4-13% of WT in developing wood (Fig. 7c; paper I, Fig. 1c). Despite of this reduction in the activity, trees developed normally (paper I, Table 1).

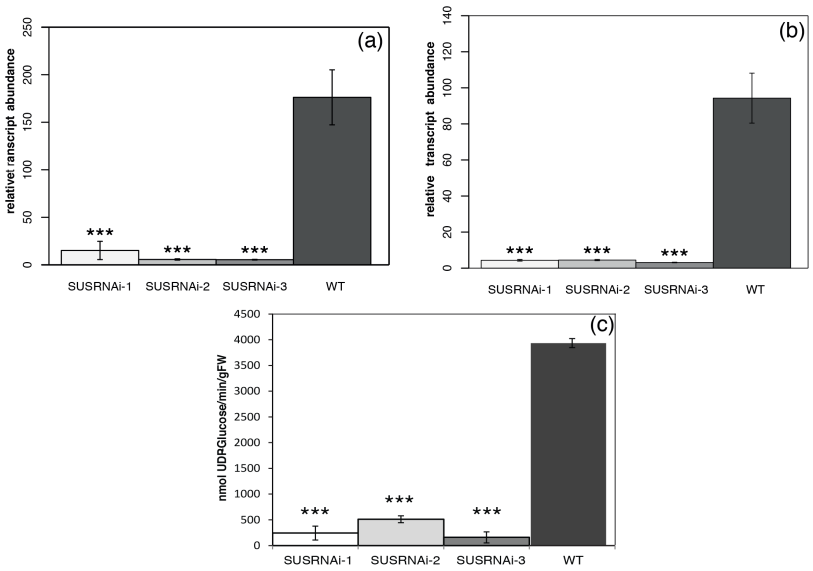

Fig. 7 *SUS1* and *SUS2* expression, and total sucrose synthase activity, in developing wood of hybrid aspen (*Populus tremula x tremuloides*) *SUSRNAi* lines. (a) Relative expression of SUS1 in wild-type (WT) and *SUSRNAi* lines 1, 2 and 3. (b) Relative expression of SUS2 in WT and *SUSRNAi* lines 1, 2 and 3. (c) SUS activity in developing wood of WT and *SUSRNAi* lines. Activity was assayed in the sucrose cleavage direction as measured by UDP-dependent UDP-glucose production at pH 7.0. Values are means _ SE. For expression values, n = 5 biological replicates; for SUS activity, n = 6 biological replicates. Student's t-test comparison with WT: ***, $P < 0.001$.

To further investigate the effect of SUS activity reduction in wood formation, both structure and chemistry of wood were analysed in mutant lines. The measurement of fiber cell wall area and wood density showed a decrease in *SUSRNAi* lines compared to WT (paper I, Table 4). Following, wet chemistry analyses revealed less cellulose, lignin and hemicellulose content per volume of wood (paper I, Fig. 3b).

To investigate the effect of reduced SUS activity in sink strength, the soluble sugar content was analysed. The mutant lines had an increase in Suc and hexose levels, but a decrease in UDP-Glc and hexose phosphates levels (paper I, Fig. 4a and 4b). This indicated no inhibition in carbon supply from phloem, however indicated a reduction in carbon flux to hexoses phosphates and UDP-Glc.

As a result, our study demonstrates that SUS has an important role in carbon flux from Suc to all wood polymers and has no essential role in supplying UDP-Glc to CSC. A similar conclusion was suggested by a study in which the

expression of mung bean (*Vigna radiata*) *SUS* in *Populus alba* did not change cell wall polymer content, but resulted in elevated incorporation of isotopically labelled Suc into cellulose and xyloglucan (Konishi *et al.*, 2004). In another study, expression of cotton *SUS* under *35S* and vascular *4CL* promoter in *Populus alba x grandidentata* increased wood cellulose and hemicellulose proportions (Coleman *et al.*, 2009). The observations from these studies suggest that an increase in SUS activity in developing wood can increase carbon allocation not only to cellulose but also to non-cellulosic cell wall polymers.

When all these studies are taken together, they imply another pathway which can also provide UDP-Glc for cellulose production. We, therefore, investigated the role of cNINs in developing wood of hybrid aspen in the next study (section 4.2).

4.2 Cellulose biosynthesis in wood relies on cytosolic invertase activity (paper II)

NINs are now included in many cellulose biosynthesis models as an alternative to SUS (McFarlane *et al.*, 2014; Endler & Persson, 2011), but so far there are no studies showing a direct evidence linking NINs to cell wall biosynthesis.

In paper II, to investigate the role of cNINs in cell wall biosynthesis, the level of *NIN* transcripts (β clade *NIN*s) was checked using available microarray data (Hertzberg *et al.*, 2001) (paper II, Fig. S2a and S2b). *NIN12* transcript was identified as the most abundant β clade *NIN* transcript during secondary cell wall formation. β clade *NIN*s were predicted to localize in cytosol (Bocock *et al.*, 2008). To experimentally determine the subcellular localization of NIN12, a yellow fluorescent protein (*YFP*)*:NIN12* fusion construct was transiently expressed in tobacco. Like YFP control, (YFP):NIN12 and NIN12:(YFP) was detected in cytosol (paper II, Fig. S3). To further characterize NIN12, a recombinant NIN12 (rNIN12) was produced in *Escherichia coli* and kinetic properties were analysed. rNIN12 showed sucrose cleavage activity at alkaline pH (pH 8-8.5) (paper II, Fig. S1b). It had a K_M for sucrose of around 7 mM at the temperature 25°C, pH 7, and was able to hydrolyse sucrose but not maltose, raffinose or trehalose *in vitro*. These results indicated that NIN12 is a typical cytosolic neutral/alkaline invertase (cNIN) (Xiang *et al.*, 2011; Qi *et al.*, 2007; Vargas *et al.*, 2007; Lee & Sturm, 1996).

In the next step, hybrid aspen *NIN12* transcript was downregulated using RNAi under the *GT43B* promoter. This promoter is active during secondary cell wall

formation (Ratke *et al.*, 2015), therefore using it would prevent pleiotropic effects due to *NIN12* transcript reduction in other tissues. It should be noted that *NIN12* has high homology (93%) with *NIN8,* which is also expressed in wood (paper II, Fig. S2a) hence neither RNAi targeting nor qPCR analysis could distinguish these two isoforms. Three lines with 60 - 66 % reduced *NIN12/8* transcript compared to WT were chosen for further investigation (Fig. 8a; paper II, Fig. 1a). Following, the reduction in transcript level resulted in reduced total soluble NIN activity in transgenic lines to 45 - 62% of WT (Fig. 8b; paper II, Fig. 1b). However, the greenhouse grown *NIN12/8-RNAi* lines showed no obvious altered growth phenotypes (paper II, Fig. S5) and no obvious changes in general wood anatomy, vessel density, or wood fiber cell wall thickness compared to WT (paper II, Fig. S6).

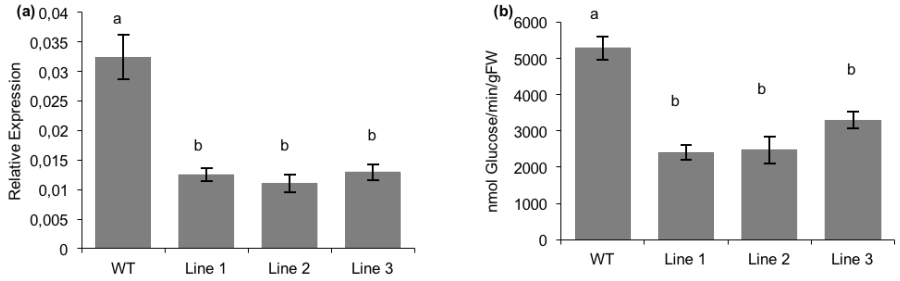

Fig 8 *CIN8/12* transcript level and total neutral invertase activity in developing wood of *CIN12RNAi* lines. **(a)** Relative transcript abundance of *CIN8/12* in WT and *CIN12RNAi* lines 1, 2 and 3. Values are means ± SE (n = 3 – 4 biological replicates). **(b)** Total neutral invertase activity in developing wood of WT and *CIN12RNAi* lines. Activity was assayed as sucrose derived hexose production at pH 7.0. Values are means ± SE (n = 3 – 4 biological replicates). Means not sharing a common letter are significantly different at P <0.001 as determined by Tukey's test after one-way ANOVA

To see the effect of reduced cNIN activity in cell wall polymer biosynthesis, analysis of cell wall composition of mature wood of *NIN12RNAi* lines was performed. Updegraff cellulose analysis, which measures crystalline cellulose, showed a significant reduction (9 – 13%) in weight proportion (Fig. 9a; paper II, Fig. 2a). Klason lignin analysis, which measures insoluble lignin content, revealed no change (Fig. 9b; paper II, Fig. 2b). Interestingly, cell wall monosaccharide composition analysis in acetyl chloride/methanol extracted wood showed a 52 – 62% increase in glucose content of transgenic lines, while hemicellulosic monosaccharides were unaffected (Fig. 9c; paper II, Fig. 2c). Cellulose microfibrils consist of a mixture of crystalline and less well organized amorphous regions (Nishiyama, 2009); the latter being soluble in acid extraction (Sannigrahi *et al.*, 2008; Bondeson *et al.*, 2006). We, therefore,

hypothesized that the high Glc content in cell wall monosaccharide composition was observed due to increased amorphous cellulose in transgenic lines.

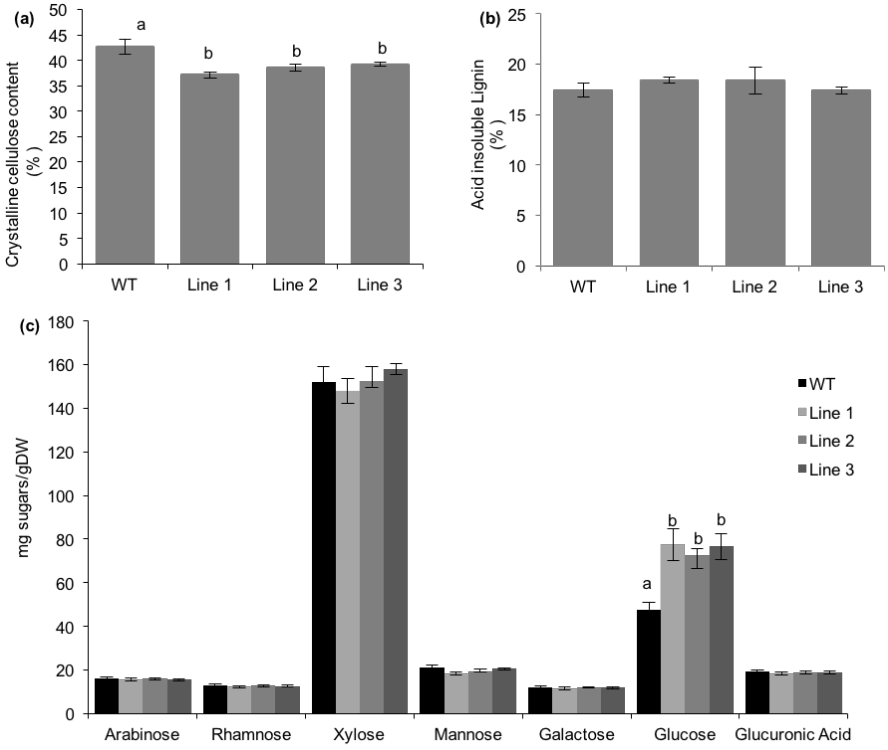

Fig. 9 Mature wood composition of WT and *CIN12RNAi* lines. **(a)** Crystalline (Updegraff) cellulose. **(b)** Acid insoluble lignin. **(c)** Sugar composition of the 2 M acetyl chloride/methanol extracted wood fraction. Values are means ± SE (n = 4 biological replicates). Means not sharing a common letter are significantly different at P <0.005 as determined by Tukey's test after one-way ANOVA.

To test this hypothesis, the enzymatic saccharification of de-starched, soluble sugar free and homogenized mature wood was performed using either cellulose specific hydrolytic enzymes or a cocktail of enzymes which also targets hemicelluloses. The cellulose specific treatment released 12-21% more glucose from *NIN12/8-RNAi* compared to WT (paper II, Fig. 3a). Consistent with this, the enzyme cocktail treatment to check any changes in both cellulose and hemicellulose digestion showed a 12-21% increase in Glc release, whereas no consistent difference was detected in hemicellulosic sugars (paper II, Fig. 3b). These results indicated that the higher Glc release in transgenic lines was most likely derived from cellulose. This supported our hypothesis that the reduction

in NIN12/8 activity altered CMF structure. This conclusion led us to investigate CMFs using scanning electron microscopy (SEM). CMF diameters of mature wood samples treated with Updegraff reagent were measured from SEM images and showed a shift in the size distribution towards thinner fibrils in all *NIN12/8-RNAi* lines compared to WT (Fig. 10; paper II, Fig. 4).

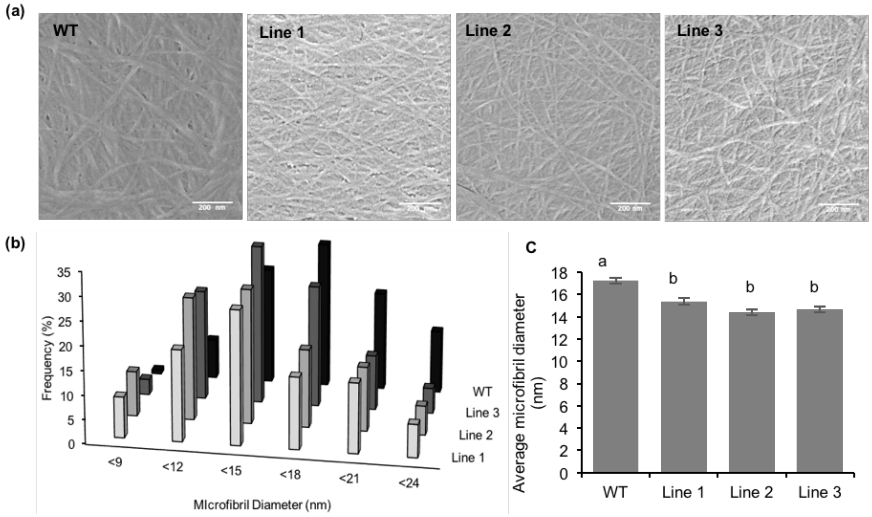

Fig. 10 Cellulose microfibril diameter of WT and *CIN12RNAi* lines. **(a)** Scanning electron microscopy images of WT and *CIN12RNAi* wood treated with Updegraff reagent prior to imaging. **(b)** Cellulose microfibril diameter distributions measured from SEM images using ImageJ software. **(c)** Average cellulose microfibril diameter. Values are means ± SE. (n = 4 biological replicates, 60 – 70 fibrils per replicate). Means not sharing a common letter are significantly different at P <0.0001 as determined by Tukey's test after one-way ANOVA.

The possible explanation for altered cellulose in transgenic lines, whose total NIN activity was reduced, could be limited carbon supply to cellulose biosynthesis. The effect of limited substrate on starch biosynthesis can be given as an example of this explanation. The reduced ADP-glucose pyrophosphorylase activity, which produces the ADP-glucose used by starch synthases (Martin & Smith, 1995), caused a reduction in amylopectin chain length distribution in the green algae *Chlamydomonas reinhardtii* and potato tubers (Lloyd *et al.*, 1999; Van den Koornhuyse *et al.*, 1996). In the next step, soluble sugar and UDP-Glc analyses were performed to check whether limiting substrate altered CMF structure. Suc, Glc and Fru levels were not significantly different compared to WT, but UDP-Glc levels were reduced to 53 – 78% of WT levels in the transgenic lines (paper II, Fig. 5). This indicated that the presence of less UDP-Glc for cellulose synthesis altered cellulose structure, which had more disorganized (amorphous) cellulose and less crystalline

structure. It can be speculated that the limited UDP-Glc caused premature glucan chain terminations during cellulose biosynthesis and therefore more amorphous cellulose were formed.

In paper II, I also investigated whether the change in cellulose was due to primary or secondary effect of the reduced NIN activity by sequencing mRNA in WT and Line 2. Surprisingly, only 2 genes were significantly up-regulated and 6 genes were significantly down regulated (paper II, Table S3). Among these differentially expressed genes, only *NIN12* and *NIN8* were related to carbohydrate metabolism.

In conclusion, the reduction in cytosolic NIN activity reduced UDP-Glc availability for cellulose biosynthesis and caused an altered cellulose phenotype. This study shows that cNINs in developing wood of hybrid aspen are critical for cellulose formation.

4.3 Determination of sugar phosphates in plants using combined reversed phase chromatography and tandem mass spectrometry (paper III)

Sugar phosphate (sugar-P) analysis is important to understand the central carbon metabolism and investigate the effect of mutations on metabolites of wood. Therefore, a comprehensive, simple and reproducible method is required to identify and quantify sugar-Ps in plant materials.

In paper III, I developed a two-step derivatization method to improve the chromatographic properties of sugar-Ps by reverse phase liquid chromatography (RP-LC), in order to identify and quantify sugar-Ps in hybrid aspen leaves and wood. The identification of metabolites by RP-LC-MS/MS depends on the data obtained from standard analysis. I, therefore, derivatized 19 authentic standards (Fig. 11; paper III, Fig. 2), first using methoxylamine (Gullberg *et al.*, 2004), and then a second derivatization was performed by application of propionic acid anhydride (Nordstrom *et al.*, 2004) and *N*-methylimidazol as catalyst. In the first derivatization step, metabolites with a free carbonyl group, such as Glc-6-P, Fru-6-P and 3-PGA, were first transformed into methoxime derivatives (-CH_3ON). In the second derivatization step, hydroxyl groups of metabolites were esterified by propionylation (Fig. 6; paper III, Fig. 1). Subsequently, the derivatized authentic standards were analysed by UHPLC-ESI-QTOF-MS, using different columns such as such as Waters Acquity HSS-T3, Phenomenex Kinetex EVO-

C18, Phenomenex Kinetex Biphenyl, Phenomenex Kinetex phenyl-hexyl, Phenomenex Kinetex pentafluorophenyl or Phenomenex Kinetex Synergi Fusion-RP C18 and mobile phases, consisting of different concentrations of water (with 0.1-2% HCOOH) and MeOH, or AcN (with 0.1-2% HCOOH), with binary gradient elution. The best separation was obtained with the Waters Acquity HSS-T3 column and MeOH as an organic mobile solvent. In addition, peak tailing was reduced by increasing the concentration of formic acid to 2% in the mobile phase (paper III, Fig. S2), where pH was lowered and consequently decreased interaction between phosphate groups and the column material.

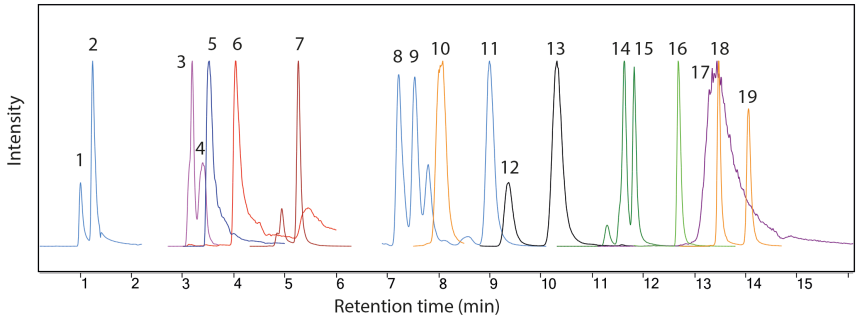

Fig. 11 UHPLC-MRM-MS profiles of sugar phosphate derivatives of a standard solution consisting of: (1) 3-PGA, 2-PGA, (3) GAP, (4) DHAP, (5) RuBP, (6) FBP, (7) E4P, (8) X5P, (9) Ru5P, (10) 2-DeoxyG (IS), (11) R5P, (12) Gal1P, (13) G1P, (14) F6P, (15) G6P, (16) Sedu7P, (17) UDP-G, (18) T6P, (19) S6P. (see the full extent of abbreviations in paper III, Table 1.)

The derivatization of metabolites and good separation on RP-LC provided an easy way to distinguish structural isomers such as Glc-1-P and Glc-6-P on MS/MS spectra (paper III, Fig. 1), and MRM-transitions were different for isomers (paper III, Fig. S1, Table S1). The results of the separation and the detection of standards by UHPLC-ESI-QTOF-MS showed that the derivatization by oximation and propionylation is an efficient method for improving the retention and the separation of sugar-Ps on RP-LC by making the polar compounds more hydrophobic.

To identify sugar-Ps in plant materials, I extracted metabolites from *Populus* leaf and wood samples by using two different procedures. The first procedure was a one-phase extraction with chloroform/MeOH/H_2O (1:3:1) mixture (Gullberg *et al.*, 2004) and the second one was the extraction with chloroform/methanol (3:7) and partitioned against H_2O (Lunn *et al.*, 2006). Since only small differences were detected between these two procedures, I used the widely accepted method, chloroform/methanol (3:7), for metabolite

extraction in further experiments. After the extraction, the derivatization method was applied and derivatives were analysed as described above. The analysis identified 18 sugar-Ps including UDP-Glc in hybrid aspen wood, based on comparison between retention time and MS/MS-spectra with standard compounds (paper III, Table 1). The mass error, which is the difference between the experimental and theoretical mass of the analyte, describes how well the analyser measures the mass, showed a range between 0.05 to 6.1 mDa. The highest mass error, 6.1 mDa, was calculated for Fru-1,6-P, which could be due to interfering substances in the corresponding chromatographic area. These results showed a good identification and mass determination of selected sugar-Ps.

There are several aspects of mass spectrometry that are important for the analysis, and quantification of metabolites, such as sensitivity, linearity and reproducibility. Sensitivity is the smallest amount of a metabolite that can be analysed in the mass spectra. Linearity is the range of analyte concentration to which ion signal is linearly related, and reproducibility refers to the precision of replicated measurements. In this study, the sensitivity and linearity were measured by using concentrations from 50 pg to 20 ng/µl of standards, which included 2-Deoxy-glucose as an internal standard (IS) (paper III, Table 2). The sensitivity measurement showed the lower limit of detection (LLOD), ranging from low to high picomoles. Compared to other studies, the sensitivity results were 10 to 1000 times better for many compounds (Arrivault *et al.*, 2009; Cruz *et al.*, 2008; Luo *et al.*, 2007; Lunn *et al.*, 2006). The linearity analysis showed a good quantitative link between the MS response and analyte concentration where R^2 was ≥ 0.99, except for 3-PGA (R^2=0.98) and UDP-Glc (R^2 = 0.97). Moreover, most of analytes were detected in the 50-5000 pg range. The precision of standards using both the 125pg and 2500pg calibration levels showed relative standard deviation (RSD %) values <9.0. In addition to standard analysis, the reproducibility of the developed method in this study applied to 10 pooled biological replicates of hybrid aspen leaf and wood, respectively. The RSD values for the majority of the sugar-Ps were 5-20% in hybrid aspen leaf and 5-15% in hybrid aspen wood (paper III, Table 2). However, RSD values for Suc-6-P was 21.1% in leaf, and 2-PGA, E4P and X5P had RSD values over 20% in wood. A reason for high RSD values could be due to the low abundance of some of these sugar-Ps and/or interferences with closely co-eluting isomeric compounds. As a result, these measurements demonstrated the high sensitivity, good linearity and reproducibility of the method.

To quantify extracted sugar-Ps from *Populus* leaf and wood, UHPLC-ESI-QqQ-MS was used. In *Populus* leaf samples, the analysis could quantify 16 out of 17 sugar-Ps ranging between 1.8-86.2 ng/mgFW. On the other hand, 12 sugar-Ps were quantified in *Populus* wood samples ranging between 2.0-143.2 ng/mgFW (Table 3). Suc-6-P in leaf samples and 3-PGA, 2-PGA, Glc-1-P, Sedu7P and Suc-6-P in wood samples could not be quantified. The concentrations of unquantified analytes did not fit the calibration curve and therefore they were considered as less than the lower limit of detection. However, this situation can be improved by extending the calibration curves and lowering the concentration of internal standard in the samples.

In paper III, I also investigated the recovery of metabolites after extraction. To check whether the metabolites of interest were quantitatively extracted during chloroform/methanol (3:7) extraction, 8 standard compounds including IS (paper III, Table 3) were derivatized either with or without performing the extraction procedure. The recovery of selected standards was between 70% and 92%, except 3-PGA (63.5%) (paper III, Table 3). The results indicated losses during the extraction procedure. To validate the method for extracted metabolites from hybrid aspen leaf, analytical recoveries of extracted samples were measured by a spiking approach of selected metabolites, where known amounts of 8 standard compounds were added to the samples during the extraction. The recovery tests were between 80-120% with RSD values of 5-14% (paper III, Table 4). The recovery test showed that all of the selected metabolites were quantitatively extracted from hybrid aspen leaf and the method could be applicable for plant materials.

In contrast to previous studies where AEC-MS/MS (Lunn *et al.*, 2006) or IPC-MS/MS (Arrivault *et al.*, 2009; Luo *et al.*, 2007) were used to analyse sugar-Ps, I was able to separate disaccharide phosphate isomers T6P/S6P and hexose phosphate isomers such as Glc-1-P/Glc-6-P together using the derivatization method and separation/detection on RP-LC-MS (paper III, Fig. S3 and Fig. S4). In addition, two unknown disaccharide phosphates, which had a retention time between Suc-6-P and Tre-6-P, were observed (paper III, Fig. S3). However, it was not possible to annotate these disaccharide phosphates, because the tandem mass spectra were identical between the different isomers, and no other standards were available.

In conclusion, the method which was developed in this part of my PhD study provided a simple, fast and robust protocol to separate, identify and quantify sugar-Ps in plant extracts.

5 Conclusion and future perspectives

Biosynthesis of cellulose and other cell wall polymers in hybrid aspen depends on sucrose metabolism. Sucrose catabolism, therefore, has a great importance in providing carbon for wood biosynthesis. Sucrose is catabolised either by SUSs or NINs after it is actively imported into the wood cells.

In paper I, RNAi mediated down regulation of highly expressed *SUS* genes during secondary cell wall formation in wood revealed that soluble SUSs have a role in carbon allocation not only to cellulose but also to non-cellulosic cell wall polymers. The findings supported studies, which challenged current sucrose synthase model where sucrose synthase is associated to the CSC and supplies UDP-glucose directly to the CSC. However, this study does not refute the possibility of CSC associated SUSs. A further investigation of CSC associated SUSs in developing wood can address the question whether associated SUSs exist and contribute to cellulose biosynthesis in developing wood of hybrid aspen. Co-immuno-precipitation analysis and SUS activity measurement in enriched cell membrane fractions in developing wood of hybrid aspen could be used to investigate the contribution of associated SUSs in cellulose biosynthesis.

In paper II, down regulation of the *NIN* genes in developing wood of hybrid aspen showed that sucrose cleavage by NINs contributed significantly to the UDP-glucose biosynthesis pathway and consequently changed only cellulose microfibril structure among other cell wall polymers. The findings in this study improved our understanding of cellulose and wood formation in trees that cellulose biosynthesis requires cytosolic UDP-glucose and it is more sensitive to shortage of UDP-glucose compared to hemicellulosic cell wall polymers. In this study, the effect of reduced cNIN activity on cellulose was modest. The presence of other cNINs and SUS, which are still expressed during developing

wood, might compensate the reduction of NIN12/8 activity and recover the effect on cellulose. Targeting also these remaining cNINs and /or SUSs by RNAi or by current methods such as CRISPR/Cas9 can create a stronger phenotype and provide better information about the effect of cytosolic UDP-glucose shortage on other cell wall polymers. Furthermore, unlike acidic INV, there are only few studies showing post-transcriptional regulation of NINs, so the field requires more investigation on regulation of cNINs and how cNIN activity is synchronized by both sucrose synthesis and import to the cell. In addition, increased glucose yield in transgenic lines by enzymatic digestion of cellulose and unchanged total cellulose content of wood opened new possibilities in cellulose microfibril engineering for biorefinery applications. As a next step, growing these transgenic lines on the field will be interesting to observe how they can adapt to environment and whether they can be grown for biorefinery purposes.

In paper III, the quantitative analysis of sugar-Ps was improved by the development of a two-step derivatization procedure combined with RP-LC-MS. The chemical derivatization strategy provided a cheap and easy way to separate sugar-Ps, including structural isomers on traditional RP-LC and increased the sensitivity of mass spectra in negative ion ESI-MS mode. This approach was the first example of chemical derivatisation of sugar phosphates where both reducing and non-reducing sugar phosphates could be analysed in a single LC-MS run. In paper III, the focus was on sugar-Ps, but this method can also be applied to organic acids and other reducing sugars. In the next step, to include analysis of more metabolites, the method should be also optimized for other compounds using their authentic standards. In addition, the method was validated using extracted metabolites only from hybrid aspen wood and leaves. Using other types of plant materials, such as *Arabidopsis* leaf, root or cell-culture extracts, can increase the validity of the method. Another issue, which should be considered in the next step, is to broaden the application of this method. For example, optimizing the analysis for isotope-labelled standards can provide the usage of this method in carbon flux analysis.

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