Elucidating the phytohormonal control of xylem development

Christoffer Johnsson
Faculty of Forest Sciences
Department of Forest Genetics and Plant Physiology
Umeå

Doctoral thesis
Swedish University of Agricultural Sciences
Umeå 2018

Acta Universitatis agriculturae Sueciae
2018:20

Cover: Composite of confocal images of three Arabidopsis (left) stem sections and three Populus (right) stem sections stained with Calcofluor white to reveal cellulose, arranged around an early illustration of Ash wood by A. van Leeuwenhoek (1632-1723).

(Photos: C. Johnsson, Illustration: A. van Leeuwenhoek)

ISSN 1652-6880
ISBN (print version) 978- 91-7760-180-7
ISBN (electronic version) 978- 91-7760-181-4

Print: Arkitektkopia, Umeå 2018

Elucidating the phytohormonal control of xylem development

Abstract

Secondary xylem, commonly known as wood, has had a major impact on both our planet and civilization, its uses so ubiquitous that it is often taken for granted in daily life.

New challenges imposed by growing population and changing climate have led academia and industries to search for ways of altering wood characteristics to better suit specific purposes. Achieving this by genetic engineering requires the ability to alter gene expression in specific cell types, while avoiding alterations in others. This calls for an in depth understanding of the factors directing the differentiation and development of the cell types constituting secondary xylem.

Studies detailed in this thesis exhibit my attempts to link the signals of the phytohormones auxin and gibberellic acid to the formation of fibres, and their deposition of thick secondary cell walls. Using high resolution RNA-sequencing data gathered across developing Populus wood, I was able to illustrate that the expression profiles of various wood-associated transcription factors are distinct, in relation to their functions. Furthermore, I investigated the response of gene expression and tissue anatomy to treatment by auxin and gibberellin and found that auxin down-regulates transcription factors previously identified as master regulators of fibre secondary cell wall formation, as well as impede secondary wall development on a tissue level. At the same time, gibberellic acid appears to have a general enhancing effect on the expression of the same genes, and is better able to rescue the tissue phenotype of decapitated Populus plants.

I also highlight a role for gibberellic acid in the differentiation of fibre cells through DELLA regulated interaction of Class I KNOX transcription factors with proteins of the NUCLEAR FACTOR Y family.

These findings suggest that an integration of auxin and gibberellin signals, acting through multiple pathways, is responsible for the correct spatiotemporal differentiation and development of the secondary xylem.

Keywords: Xylem, auxin, gibberellin, differentiation, secondary cell wall

Author's address: Christoffer Johnsson, SLU, Department of Forest Genetics and Plant Physiology,
Swedish University of Agricultural Sciences, 90183 UMEÅ, Sweden

Dedication

To my late grandmother, Aino, whose example I can only hope to emulate.

Sometimes science is more art than science… A lot of people don't get that.
R. Sanchez

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 Christoffer Johnsson, Urs Fischer* (2016). Cambial stem cells and their niche. *Plant Science*, 252 (2016), pp. 239-245.

II David Sundell, Nathaniel R Street, Manoj Kumar, Ewa J Mellerowicz, Melis Kucukoglu, Christoffer Johnsson, Vikash Kumar, Chanaka Mannapperuma, Nicolas Delhomme, Ove Nilsson, Hannele Tuominen, Edouard Pesquet, Urs Fischer, Totte Niittylä, Bjorn Sundberg, Torgeir R Hvidsten* (2017). AspWood: High-spatial-resolution transcriptome profiles reveal uncharacterized modularity of wood formation in Populus tremula. *Plant Cell*, 29 (7), pp. 1585-1604

III Christoffer Johnsson, Xu Jin, Weiya Xue, Carole Dubreuil, Lina Lezhneva, Urs Fischer* (2018). The plant hormone auxin directs timing of xylem development by inhibition of secondary cell wall deposition through repression of secondary wall NAC-domain transcription factors. (Accepted)

IV Christoffer Johnsson, Lina Lezhneva, Daniela Liebsch, and Urs Fischer*. Class I KNOX transcription factors interact with the NF-FY complex to promote fibre differentiation in the hypocotyl (manuscript)

* Corresponding author.

The contribution of Christoffer Johnsson to the papers included in this thesis was as follows:

I Writing, editing and formatting the manuscript.

II Analysis and interpretation of the data, writing of the manuscript.

III Planning and performance of experimental work, data analysis and interpretation, writing and preparation of the manuscript.

IV Planning and performance of experiments, data analysis and interpretation, writing and preparation of the manuscript.

Abbreviations

ARF	auxin response factor
bHLH	basic helix-loop-helix
bp	base-pair
CESA	cellulose synthase
ChIP	chromatin immuno-precipitation
CLE	clavata3/esr-related41
CSC	cellulose synthase complex
FSC	forest stewardship council
GA	gibberellic acid
GE	genetic engineering
GID	gibberellin insensitive dwarf
GM(O)	genetically modified (organism)
GUS	β-glucuronidase
IAA	indole-3-acetic acid
IRX	irregular xylem
KNAT	knotted-like from arabidopsis thaliana
KNOX	knotted1-like homeobox
LAC	laccase-like
LRR-RLK	leucine rich repeat receptor-like kinase
MYA	million years ago
MYB	myeloblastosis
NAA	1-naphthaleneacetic acid
NAC	nam, ataf, cuc
NF-Y	nuclear factor y
NST	nac secondary wall thickening promoting factor
PCD	programmed cell death

PIN	pin-formed
POM2	cellulose synthase-interactive protein 1
PXY	phloem intercalated with xylem
RAM	root apical meristem
SAM	shoot apical meristem
SCF	skp, cullin f-box
SCW	secondary cell wall
SND	secondary wall-associated nac domain
STM	shoot meristemless
SWN	secondary wall nac
TF	transcription factor
VND	vascular-related nac domain
WOX	wuschel-related homeobox

1 Introduction

1.1 The evolution and importance of wood

The establishment of multicellular plant life on land, some 470 million years ago (MYA) (Kenrick et al. 2012), marks the beginning of a new evolutionary arms race. Competition for light drove plants to out-grow their neighbours by reaching for the sky, something that brought with it several novel challenges. Firstly, water had to be transported from moist soils to the sites of photosynthesis, a process which initially was possible through evapotranspiration-driven water movement through cell walls themselves, and secondly; plants needed to support their own weight against gravity when no longer held up by the buoyancy of water, before moving on to land, or by turgor pressure after first making the move from an aquatic environment (Sperry 2003). The earliest evidence of specialised water-transporting tissues has been described in fossils of the genus *Cooksonia* (Edwards et al. 1992) dating from 433-393 MYA, where the stem is described as containing a central vascular cylinder, reminiscent of a transitional form between non-vascular bryophytes, such as liverworts, and vascular plants. Reinforced tracheids which could withstand greater water tension were an improvement on those first vascular cylinders and allowed vascular tissues to transport water over greater vertical distances. Water conductivity is dependent on conduit diameter. However, the size of a tracheid is limited, as larger diameters increases the risk of cavitation and subsequent embolisms, and also affect frost sensitivity (Christensen-Dalsgaard & Tyree 2014). This limit to water transport capacity was overcome, in part, by the evolution of hollow end-joined vessel elements, which allowed multiple cells to form long, wide, water conduits. This specialized cell type led to the plant devoting less of its cross-sectional area to water transport, while allowing for non-vessel tracheid cells to evolve into

fibres, gaining stronger cell walls but largely losing the ability to conduct water.

Individual fibres were longer than vessel elements and imparted greater mechanical strength to woody tissues, allowing plants to reach greater heights, and withstand harsher environments. Additionally, fibres provided a greater resistance to pathogens and pests thanks to the increased proportion of indigestible wood polymer. The chief components of the thick secondary cell walls (SCWs) of both vessels and fibres are cellulose, lignin and hemicelluloses. These are produced and laid down by a large number biosynthetic and other enzymes, under the control of a network of transcription factors (TFs), a process starting with the division of stem cells in the cambium. Cellulose is the most abundant biopolymer on Earth, commonly comprising as much as 40-45% of oven-dried wood biomass in softwoods and reaching as much as 50% in hardwoods (Ragauskas n.d.). Cellulose polymers are formed from β1-4 linked d-glucose monomers and can often reach a length of several thousand units (Klemm et al. 2005). Multiple such polymers then aggregate to form crystalline cellulose. Interactions between cellulose microfibrils and hemicellulose act to connect the microfibrils to the cell wall polymer lignin. Lignin is a complex, branched polymer consisting of phenolic monolignol subunits and forms a rigid matrix in spaces between other cell wall polymers, and also acts as an insulator against water permeation by virtue its hydrophobic properties. Monolignol composition varies between species, but the function of the polymer remains the same: to reinforce the cell wall against mechanical stresses and degradation by pathogens and pests. Not to be forgotten; the impregnability of the secondary cell wall to water is also an important factor allowing vertical water transport to take place, by ensuring that vessels do not leak, and can thus maintain their negative pressure throughout the plant.

It is the diverse chemo-mechanical properties of wood that have allowed humans to employ it for a multitude of purposes throughout history; from burning (i.e. fuel), to structural material in furniture and homes, to tools, paper, and most recently; carbon fibre and other biomaterials. However, the properties of wood which are beneficial for a tree from an evolutionary standpoint, i.e. increasing the fitness and survivability of an organism and its offspring, are not necessarily optimal for human needs and purposes. A classic example is the recalcitrance to degradation faced by the pulp industry when attempting to separate lignin and cellulose during bleached pulp production. Research has shown that lignin is essential for proper plant growth and development, making it difficult to reduce its content through breeding or genetic engineering (Eudes et al. 2014) for reasons related to yield and survivability of the resulting plant, rather than the success rate of the techniques themselves. Traditional breeding

of non-fruit trees is a fairly recent concept, compared to other crop species, with the first serious efforts arising during the 20th century. Even though the better part of a century has been spent improving both qualitative and quantitative traits in a number of species (primarily Eucalypts spp., *Pinus radiata* and *Pinus taeda*, and *Picea abies*), the long generation times of flowering trees mean that in species where clonal propagation and flower induction are not an option, only 3-5 generations have been through the programmes to date. Compounding this, research into the genetics of trees over the past decades has illustrated that many traits are complex, i.e. either governed directly by a large set of genes, or closely linked to multiple genes, making it difficult to select for superior trees among a population to act as parents for the next generation.

The ability of trees to sequester carbon from the atmosphere has played an important role in the history of our planet, particularly prior to the evolution (Floudas et al. 2012) of wood-degrading micro-organisms, as carbon stored in tree trunks was trapped underground and eventually fossilised to form coal deposits. And in part due to the current debate on climate change, the enzymatic processes that have evolved to degrade wood in natural systems are being researched with the goal of harnessing them for the industrial degradation of lignocellulosic biomass and the production of valuable products from waste streams, with the aim of decreasing the requirement for energy and chemicals in these processes.

Conversely, forests have also been suggested to once again be used as carbon sinks, with large scale plantation programmes (Oliver 2018) able to sequester massive amounts of atmospheric carbon for long periods of time. This would require use of local species, or breeding/engineering of fast-growing species (e.g. Eucalypts) to adapt them to local climates to optimise growth. Another proposed method to exploit wood as carbon sink involves using more timber in construction. This is a concept that has gained considerable attention in recent years, as projects such as the proposed "Oakwood Timber tower" (Architecture 2017) helped put the spotlight on the potential of improved wood-based materials such as cross-laminated timber as a replacement for steel and concrete. While tree breeding has made great strides over the past century, the problems described illustrate the challenges faced when using this method along. Genetic engineering could potentially alleviate some of the problems associated with sub-optimal wood properties. However, biotechnological methods must be applied on pre-selected and characterised elite lines, to properly target alterations and minimise the risk of unforeseen adverse effects. Given that quantitative traits such as growth have been the main focus of most traditional breeding programmes to date, it is

likely that future genetic engineering efforts will be directed toward altering qualitative traits, such as lignin composition (or structure), pest and pathogen resistance, or resistance to abiotic factors.

Recent advances in applied biotechnology, in the form of genetic engineering (GE), by private companies have claimed to increase the growth of both soft- and hardwood species[†], but have yet to be widely adopted due to both legal restrictions as well as resistance from non-governmental organisations e.g. certification organisations such as the Forest Stewardship Council (FSC), and public resistance to genetically modified (GM) crops. In practice, this means that even though there may be technical solutions to certain challenges and even legal permission to apply it, it may not be commercially viable due to a resulting loss of, for instance, environmental certification of the product. And it has even been alluded to that employment of GM trees may lead to dissociation of an entire company from a certain certifying organisation, as the FSC has warned would be the consequence, should Brazilian pulp and paper producer Suzano chose to deploy its approved GM Eucalypt (FSC 2015) for commercial use in Brazil. Not only do academics and corporate researches face regulatory obstacles, but this soft banning of products leads to companies being unwilling to invest in what could prove to be very useful products.

Novel technologies, such as those based on CRISPR/Cas9 offer means to circumvent some regulations as the products of these technologies may be indistinguishable from organisms produced by conventional breeding. For instance the Swedish board of agriculture has ruled that CRISPR/Cas9-edited plants may be grown in field trials without special authorisation needed for other GM organisms (GMO) (Klarin 2015), though commercial application is still not allowed.

Irrespective of method; any attempt at genetic engineering of qualitative or quantitative wood traits, e.g. altering the rate or ratio of wood deposition, requires in-depth knowledge of the molecular processes involved in the differentiation of secondary xylem, maturation of its constituent cells, and associated secondary cell wall deposition. Not only is there a need to understand the enzymes involved in cell wall biosynthesis, but also the upstream transcription factors responsible for regulating their expression, as well as a need for the regulators of the transcriptional master switches to be identified and characterised. In this thesis, I will describe my work in attempting to increase our understanding of the factors regulating the formation of secondary cell walls, and the search for a way to affect changes in wood attributes by genetic engineering methods.

† Futuragene Inc., Arborgen Inc. Private communication

1.2 Meristems

All growth in higher plants originates from two apical meristems, located in the root (Root Apical Meristem, RAM) and shoot (Shoot Apical Meristem, SAM). These meristems contain populations of un-differentiated stem cells which continuously divide to give rise to daughter cells, some of which subsequently differentiate into the below or above ground tissues, respectively, while others retain their meristematic identity. Apical meristems initially give rise to several primary meristems, from which tissues such as the epidermis, cortex, pith, and primary phloem and xylem develop. Additionally, apical meristems also produce progenitors of the secondary (lateral), meristems; the vascular cambium and cork cambium. While the cork cambium produces periderm, replacing the epidermis, derivatives of the vascular cambium differentiate to form secondary xylem and phloem, of which this thesis will treat mainly the secondary xylem. The vascular cambium, also known as the cambial meristem, contains the undifferentiated population of stem cells, transient amplifying pluripotent cells, and niche cells, which define the boundary of the different cell populations, reviewed by Johnsson & Fischer (2016). Use of the term “stem cell” in plants has been widely contested, as it originates from animal research and was borrowed in spite of the glaring differences between totipotent and pluripotent cells in the two fields. Therefore, a short clarification as to their definition is in order. Stem cells are non-differentiated cells with the ability for self-renewal and subsequent differentiation to supply the cells necessary for development of tissues and organs. While somatic stem cells in animal systems are able to migrate through the organism, this is not the case in plants, as cells are rigidly held together by intercellular linkages. Stem cells in the cambial zone have been described in various tree species, based on histology and anatomy, however, it is still unknown whether or not this stem cell population can be considered constant (as in the SAM and RAM) or if there is gradual replacement (Esau et al. 2009). Studies where agrobacterium have been utilised to induce transgenic changes directly in the cambial zone using a technique known as induced somatic sector analysis (Creux et al. 2013) have indicated that the cambial stem cell population consists of a single-cell layer of stem cells, surrounded by pluripotent stem cell derivatives, which give rise to files of phloem and xylem cells respectively. The same method also illustrates the longevity of these stem cells, as transgenic cell files stretch along a large portion of the radial distance from pith to bark. Divisions of the cambial stem cells are mainly parallel to the bark of the tree, i.e. periclinal, ensuring that stem cell cylinder integrity is maintained as the radius increases (Brackmann & Greb 2014). Radial growth results from stem cells dividing asymmetrically, maintaining the stem cell

population and leading to one daughter cell being added distally to the stem cell layer for each such division.

As to the question whether or not derivatives of anticlinal cambial stem cell are toti- or pluripotent, it is yet one more example where use of nomenclature from the animal field causes confusion when applied to the study of plants. The definition of totipotency is the ability for the derivative of a stem cell to differentiate into all the cell types of the whole organism. The term itself was coined by *Drosophila* geneticist Thomas Hunt Morgan (Laimer & Rücker 2003), and while the totipotency definition holds true for e.g. all cells in the human embryo, up until the 2-4 cell stage, after that, certain cells lose the potential for developing into extra-embryonic tissues, such as placenta (Condic 2014), and enter a state called pluripotency. While some plants are able to regrow from isolated tissues, or even single cells (Ikeuchi et al. 2016), such growth does not lead to regeneration of an endosperm, emphasising that such original cells are indeed pluripotent rather than totipotent.

Cambial stem cell derivatives only differentiate into a small number of different cell types within the cambial niche. Evidence from *Arabidopsis thaliana* (Arabidopsis) has shown that xylem cell files of hypocotyls consist of only parenchymatic and fibre cells, while some also have vessel cells (Liebsch et al. 2014). It is still currently unknown whether this difference is an effect of variation in micro-environmental cues, or points to a difference in potency of the various cell file mother cells. Supporting for the prior hypothesis comes from observed expression of vessel cell fate-determining factors VASCULAR-RELATED NAC DOMAIN 6 and 7 (VND6/7) in both vessels and fibres (Obudulu et al. 2016), which would seem to indicate that all cells are initially primed for a vessel cell fate, but later receive another signal leading to most cells adopting a fibre cell fate as they leave the niche.

1.3 The cambial zone

The cambial zone consists of the stem cell layer, surrounded by what is known as the cambial niche. The loosely defined boundaries of the niche are thought to be regulated by the integration of both cell-intrinsic (lineage) factors and external signals, such as phytohormones. Due to the lack of mobility of plant cells; it is assumed that external factors are chief among directing these (Bhalerao & Fischer 2014). For a review of the cambial niche, see Paper I of this thesis (Johnsson & Fischer 2016). Divisions of meristematic cells in the cambial zone introduce new daughter cells to the cambial niche, while some daughter cells remain without, and assure that the pool of undifferentiated stem cells is maintained. As stem cell divisions proceed, older daughter cells are

pushed out of the niche and may undergo further differentiation, resulting in a loss of pluripotency (Chen et al. 2013) dependent on the new microenvironment in which they find themselves.

Currently, mechanisms for the direction of cambial cell division orientation, i.e. directing the balance between self-renewal and additive growth, remain unknown. Recent advances in our understanding of the direction of SAM morphogenesis may lead to new ways of studying these (Gruel et al. 2016). It has also been suggested that mechanical signals are responsible for directing the expression domain of the homeodomain transcription factor SHOOT MERISTEMLESS (STM), and also demonstrated that, in the SAM, auxin depletion is not strictly required for induced expression of STM.

Maintenance of meristematic cells in the shoot apex relies on the CLAVATA/WUSCHEL pathway, as well as the complementary action of STM (Gallois et al. 2002; Lenhard et al. 2002). While this factor, along with its close homolog BREVIPEDICELLUS (BP), also known as KNOTTED-LIKE FROM ARABIDOPSIS THALIANA (KNAT1) are expressed in both the cambial and shoot meristems, they appear to have diverged functionally. This is supported by recent data, which suggests they perform opposite functions in the respective meristems; i.e. STM and KNAT1 are necessary for proper differentiation of cambial xylem derivatives, as shown by Arabidopsis single and double mutants of these genes having reduced numbers of xylem fibre cells, while maintaining proper vessel differentiation and only displaying a mild decrease in the total number of cells (Liebsch et al. 2014). This is in contrast to their function in the SAM, where they act to direct maintenance of the undifferentiated stem cell pool.

While hormonal signals such as auxin, gibberellin and cytokinin primarily define the lateral extent of the niche, the radial dimension of the cambial zone has been proposed to be limited by a system of mobile short peptides and their receptors. The short peptide CLAVATA3/ESR-RELATED41 (CLE41), and possibly CLE44 (Hirakawa et al. 2008; Etchells & Turner 2010), are secreted in developing phloem cells, and recognized by membrane bound LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE (LRR-RLK)-family protein PHLOEM INTERCALATED WITH XYLEM (PXY), which serve to regulate gene expression through the WUSCHEL-related HOMEOBOX4 gene, WOX4 (Hirakawa et al. 2008). This leads to the formation of a boundary where the two signals meet. The topic has been recently reviewed (Etchells et al. 2015) and while very interesting, is beyond the scope of this thesis.

1.4 Secondary xylem formation

The secondary xylem, commonly known as wood, is formed from derivatives of stem cells in the vascular cambium. In angiosperm trees, such as Populus spp., the main cell types of the secondary xylem are fibres, vessels, and ray cells, of which fibres are the most abundant by far. As daughter cells of the vascular cambium are separated from the continuously dividing cambial zone, they are deprived of the signals directed at maintaining their undifferentiated state. Initially, this causes a few evenly distributed cells in the outer layers of the xylary cylinder to rapidly expand, before depositing a reinforced SCW, and finally undergoing programmed cell death (PCD), whereupon the cell is emptied and a vessel element is left behind. Surrounding cells maintain a parenchymatic function and appearance, as they expand during this process, indicating that at least part of the signal directing differentiation of vessels is of apical origin rather than from the adjacent cambium. The role of auxin influx and efflux carriers in the direction of this vascular bundle patterning has been well established (Fàbregas et al. 2015). These parenchymatic cells subsequently initiate the SCW biosynthetic program (described in more detail below), gradually forming thick SCWs (at a rate lower than in vessels) becoming what is commonly referred to as fibres.

1.5 Deposition of secondary cell walls

Deposition of SCWs requires the coordinated action of a large set of diverse proteins. The end result of their action is the production and deposition of the main components of the secondary cell wall: cellulose, lignin and hemicelluloses (e.g. xylans). It is important to note that the proportions of these may vary between species and, even between cell types within an individual plant. Typically, 40-50% of the SCW is made up of cellulose microfibrils comprised of long β-1,4-linked glycosyl residues, often reaching degrees of polymerisation in the order of 10^4 (Timell 1967) with each fibril being approximately 1.5-3.5 nm in diameter (Klemm et al. 2005). The exact number of chains in each microfibril bundle has been a matter of some debate, with the common consensus thought to be 36 microfibrils (McFarlane et al. 2014), though a recent model (Newman et al. 2013) suggests that 18 glucan chains corresponds best to the average 3 nm diameter of single primary cell wall glucan chains. Additionally, a study in spruce suggests that microfibrils may consist of 24 glucan chains (Fernandes et al. 2011).

Microfibril biosynthesis is carried out by plasma membrane-localised cellulose synthase complexes (CSCs), which were revealed by transmission electron microscopy to take on a rosette-like conformation (McFarlane et al.

2014) of six domains, each of which are proposed to consist of three CELLULOSE SYNTHASE (CESA) subunits according to the aforementioned 18-glucan chain model. All 18 subunits of the complex would be predicted to polymerise and extrude one glucan chain that is subsequently hydrogen-bonded together with its immediate neighbours to form a microfibril. However, given the studies referred to above, it is plausible that variation in CSC subunit makeup exists (from 18-36) between species and possibly also cell types, leading to correspondingly varied cellulose microfibril thickness. Synthesis of the β-1,4-glucan chain utilises UDP-glucose as a substrate in a process that forms an acetal link between each glucose ring (for a review of the function of these glycosyltransferases see Lairson et al. (Lairson et al. 2008) The translocation of the glucan chain by the CSC is thought to provide the energy for the movement of the membrane-bound CSC through the plasma membrane (Morgan et al. 2012). Studies of fluorescently tagged primary wall CESAs have indicated that the speed at which the complex moves is between 200-350nm/min (Schneider et al. 2016) and while this is in line with theoretical estimates (Schneider et al. 2016), measurements of similarly tagged secondary wall CESAs suggest that these move faster, although the study also describes how speed varies across different developmental stages (Watanabe et al. 2015). Movement of the CSC has been found to follow the cortical microtubules, via interactions with CELLULOSE SYNTHASE-INTERACTIVE PROTEIN1 (POM2) (Bringmann et al. 2012). This is likely regulated by a multitude of factors, including availability of substrates, energy, as well as stoichiometry of protein complex constituents.

As glucan chains are extruded from the CSC they spontaneously crystallise together to form microfibrils, which are subsequently coated in the additional cell wall components; lignin and hemicelluloses. The degree of crystallinity of these microfibrils greatly affects their mechanical and chemical properties, in particular its recalcitrance to degradation by enzymatic and chemical means (Nishiyama 2009).

Lignin is primarily composed of three monolignol subunits: p-coumaryl, sinapyl and coniferyl alcohol and forms a complex, seemingly random, three-dimensional mesh structure in the volume between cellulose microfibrils. Named for the chief constituent monolignols; Syringyl (S-lignin, sinapyl alcohol), Guaicyl (G-lignin, coniferyl), p-hydroxyphenyl (H-lignin, p-coumaryl) alcohol, the ratio between these types varies between species, with gymnosperms lacking S-lignin and dicots containing both S and G, while H-lignin is particularly enriched in grass species (Zhong & Ye 2015). Monolignols are derived from hydroxycinnamoyl-CoA esters produced from

phenylalanine through the phenylpropanoid pathway. A recent review describes this thoroughly (Ye & Zhong 2015).

How cytosol-synthesised monolignols are transported to the apoplastic space is still poorly understood, though one model suggests that storage and transport of monolignols utilises glucoside forms of each subunit, which are later mobilised for polymerisation through the action of a glucosidase (Freudenberg 1959) and export across the plasma membrane appears to utilise Golgi-like vesicles (Pickett-Heaps 1968). A more recent study challenges this mode of transport, showing that protein synthesis inhibitors are sufficient to remove the signal from radiolabelled monolignols observed in vesicles by previous investigators (Kaneda et al. 2008). Furthermore, there is strong evidence that export of the monolignol p-coumaryl alcohol is mediated by a plasma membrane-bound ABC-transporter in Arabidopsis (Alejandro et al. 2012).

Upon export, monolignols are polymerised in the apoplastic space, putatively by the action of laccases and peroxidases. Although the evidence for this hypothesis mainly originated from biochemical experiments *in vitro* (Bonawitz & Chapple 2010), recent data from Arabidopsis suggests that altered expression of *LACCASE-LIKE 15* (*LAC15)* results in decreased lignin deposition in the seed coat. Furthermore, disruption of the LAC4 and LAC17 enzymes affect lignin deposition in Arabidopsis interfascicular fibres (Berthet et al. 2011), confirming that this model does have some merit. Research in spruce has also implicated peroxidases as integral to proper monolignol radicalisation (Fagerstedt et al. 2010). It is this radicalisation that allows for the formation of a random polymer structure, often described as akin to chicken wire. The lignin structure imparts strength and rigidity to the cell wall, but also contributes to water transport and retention characteristics, and resistance to pathogens by providing a recalcitrant barrier against invasion of the cell.

Attempts at altering lignin composition and structure have yielded promising results, although early efforts often led to negative effects on growth (Petersen et al. 2012; Bonawitz & Chapple 2013), due mainly to impaired water transduction as weakened cell walls lead to vessel cell collapse. More recent studies have illustrated the ability of integrating non-native monolignols and changing the inter-molecular bonds between subunits to improve degradability without negatively affecting yield (Wilkerson et al. 2014; Eudes et al. 2014).

The third main component of SCWs is hemicelluloses, the most abundant of which is xylan. Xylans are synthesised in the Golgi, and consist of a backbone with an average of 120 (Jacobs & Dahlman 2001), β-1-4 linked xylosyl residues, with some residues substituted with α-1, 2-linked 4-O-

methylglucuronic acid (MeGlcA). A common attribute of xylan chains throughout woody angiosperms and gymnosperms is the presence of a unique reducing-end tetrasaccharide sequence (Zhong & Ye 2015). Early transcriptome profiling in Populus orthologues of Arabidopsis IRREGULAR XYLEM 7 (IRX7), PARVUS and IRX8 have shown them to be involved in the synthesis of this reducing end (Djerbi et al. 2005; Lee et al. 2009a; Lee et al. 2009b). Biosynthesis of the xylan backbone itself in the wood-forming zone of Populus has been suggested to involve a large set of genes, including xylosyltransferases (e.g. IRX10/IRX10L orthologues), xylan biosynthetic genes (e.g. IRX15/IRX15L orthologues), and finally a set of genes for affecting backbone modifications, including GLUCURONIC ACID SUBSTITUTION OF XYLAN (GUX) for adding GlcA onto xylan, and REDUCED WALL ACETYLATION (RWA) responsible for correct acetylation of the polymer.

1.6 Transcriptional regulation of SCW formation

Deposition of the secondary cell wall requires the spatiotemporally coordinated expression of a large set of biosynthetic genes. This has been found to be under the control of a complex hierarchical network of transcription factors, mainly of the MYELOBLASTOSIS (MYB) and NAM, ATAF, CUC (NAC)-domain transcription factors families. At the base of the network the various biosynthetic genes necessary for production of the biopolymers of the SCW themselves can be found. The intermediate level is composed of a large number of TFs regulating the expression of the biosynthetic genes directly. This level is mainly comprised of MYB TFs, which are able to activate either all three cell wall-component biosynthetic pathways (i.e. cellulose, lignin and hemicellulose) in a semi-promiscuous way, as binding elements for many of these TFs are present in promoters of a multitude of the target genes, but also includes intermediate TFs involved in the regulation of specific downstream genes, e.g. proteins repressing SCW formation such as WRKY12 (Wang et al. 2010). For an excellent review of this process, see Ye & Zhong (2015). At the top of this hierarchy lie the so-called master level transcription factors; TFs involved in direction of xylem cells differentiation, or in initiation of the transcriptional cascade. Previous research has described the master level TFs as belonging to two or more, subgroups depending on their cell type specificity. These are chiefly the VND or SND / NST1 classes, associated with vessel or fibre cells respectively. These factors regulate the expression of the mid-level TFs, but can also target some biosynthetic genes directly.

1.6.1 NAC domain transcription factors

The NAC TF family is one of the largest characterised in plants, with at least 117 members (Nuruzzaman et al. 2010) in Arabidopsis, and more than 160 in Populus (Liu et al. 2014; Hu et al. 2010). The large number of NAC proteins encoded by the genomes of both Populus and Arabidopsis predicts tremendous potential for specialisation, and it is well established that NAC TFs are involved in a plethora of developmental processes (Hu et al. 2010) Although only a small proportion of these proteins have been characterized with regard to their function, there are plenty of examples in literature of NAC TFs involved diverse processes, such as SAM function (Takada et al. 2001), vascular and flower (Sablowski & Meyerowitz 1998) development (Kubo et al. 2005), and secondary cell wall formation (Zhong et al. 2010), to name only the ones most relevant to this thesis. A typical NAC TF contains a highly conserved N-terminal NAC domain, usually of approximately 150-160 amino acids, divided into several sub-domains (Hu et al. 2010). A 60 amino acid region of this domain has been described as containing the actual TF fold, responsible for DNA binding. Apart from DNA binding functionality, the NAC domain is involved in hetero- and homodimerisation, as well as nuclear localisation (Olsen et al. 2005). The C-terminus of NAC TFs is more divergent, both in amino acid sequence and function. Most common is that it consists of a transcriptionally regulating domain, either an activator or repressor, while some NAC TF C-terminal domains also contain transmembrane motifs (Shao et al. 2015).

Phylogenetic study of NAC TFs in Populus, Arabidopsis, and rice has revealed that TFs involved in distinct processes sub-cluster together across species into 18 distinct families (Hu et al. 2010). Most of these contain genes from several species; however, there are groups that illustrate the divergence between monocots and dicots, and also between herbaceous and woody dicots. NAC genes in Populus are distributed in 19 linkage groups (across the same number of chromosomes) in a non-random fashion, indicating that even though a large number of NAC genes have overlapping expression patterns duplicates have been retained. This indicates that multiple copies have conferred a selective advantage, irrespective of whether or not both proteins retain their original function, or have taken on new regulatory roles (Hu et al. 2010).

One of the 18 subclusters includes NACs involved in the direction of SCW deposition. These secondary wall regulating NAC domain TFs, often labelled Secondary Wall NACs (SWN), have been found to direct gene expression through binding to short 19 base pair (BP) secondary wall NAC-binding element (SNBE) motifs (Zhong et al. 2011). The fact that many NAC target genes present in the network regulating SCW deposition also contain

secondary wall MYB-responsive elements (SMREs) has led to the suggestion that TFs from multiple levels of the regulatory network act in concert with each other to direct gene expression.

1.6.2 MYB domain transcription factors

MYB transcription factors are prolific throughout Eukaryotes and are, like NACs, involved in transcriptional regulation of a wide range of developmental and metabolic processes including stress response, hormone signalling, regulation of differentiation, and, most relevant to this thesis; direction of phenylpropanoid biosynthesis (Roy 2016). First described in *Zea mays* (Paz-Ares et al. 1987), large families of MYB proteins have been identified in several plant species. These families are sub-classified dependent on the quantity of repeated motifs present in the DNA-binding MYB domain. These repeats result in a structure containing 3 α-helices and consist of some 52 amino acids, and commonly occur one to four times, leading to the classification 1R, R2R3- 3R, and 4RMYB proteins, of which the R2R3 is the most common in plants (Roy 2016). Two helices of each three-helix repeat form a helix-turn-helix structure while the third constitutes a so called recognition helix, interacting directly with DNA through intercalation.

Investigation of sequence conservation within the (usually) N-terminal MYB domain and variable C-terminal domains of studied MYB TFs has led to further sub-classification of R2R3 MYB TFs. Initially based on Arabidopsis MYBs classifications, these groups have been expanded when new R2R3 MYBs, which lack Arabidopsis orthologues, have been found in additional species such as Populus.

While the sequence, structure and function of the MYB domain is highly conserved, other parts of MYB proteins are highly variable, allowing MYB TFs to carry out both transcriptional activation and repression, thus directing a wide range of cellular processes (Dubos et al. 2010).

Regulation of MYB activity occurs at several levels. A number of MYB transcripts are targets of miRNAs (Allen et al. 2007), as well as trans-acting (*ta*)-siRNAs. Post-translational modifications, e.g. phosphorylation also play an important role (Araki et al. 2004), as do interactions with other proteins (e.g. hetero- or homodimerisation), the most intensely studied of which is likely that between R2R3-MYBs and basic helix-loop-helix (bHLH) proteins (Dubos et al. 2010). A particular motif in the R3 helix of the MYB domain has been implicated in mediating this interaction, though it is not present in all R2R3 MYBs and a level of redundancy must therefore be assumed. Heterodimerisation of R2R3 MYBs with bHLH proteins is an example of

negative regulation, as binding to bHLHs sequesters MYB TFs from other interactions (e.g. homodimerisation) and does not bind DNA directly (Dubos et al. 2010).

The plurality of MYB transcription factors means that there is ample opportunity for specialisation, either through specific interaction partners, unique binding affinities, or upstream control of their expression. One example of such specification is the transcriptional regulation of secondary cell wall biosynthetic genes by binding of PtMYB2 and PtMYB20 to SMREs. A large number of MYB-family TFs have been identified to as involved in the regulation of SCW formation (Ye & Zhong 2015). For instance, MYB103 has been found to be a key regulator of S-lignin biosynthesis (Ohman et al. 2012), while an orthologue of AtMYB43 (PtrMYB152) has been found to affect xylem fibre cell area (Hussey et al. 2011), and a multitude of others have been linked to various regulatory pathways during SCW formation (Zhong et al. 2008).

1.6.3 KNOX transcription factors

KNOTTED1-like Homeobox (KNOX) genes encode a small family of plant transcription factors that was first isolated from *Zea mays* (Vollbrecht et al. 1991). These three-amino acid loop extension (TALE) proteins are classified in two groups (Class I/II) of four genes each in Arabidopsis, on the basis of homeodomain sequence similarity, phylogenetic analysis, and intron position, with proteins of higher similarity to Kn1 designated Class I KNOX proteins. Genes of this group typically also have strict expression domains, restricted to the SAM (in both monocots and dicots) while expression patterns of genes within Class II are more varied (Zhong et al. 2008) and their functions less well characterised. The general structure of a class I KNOX protein in consists of an N-terminal MEINOX domain, responsible for protein dimerization, and a C-terminal homeodomain, which binds to DNA.

Class I KNOX genes in Arabidopsis are involved in a range of developmental regulatory processes, but their main function appears to be maintenance of the SAM (Endrizzi et al. 1996), as well as control of differentiation in the cambium (Liebsch et al. 2014).

Expression of the *STM* gene in the SAM bounds the extent of that meristem, and its expression during embryogenesis is also the first measured instance of KNOX gene expression (Long et al. 1996). At a later stage, partially overlapping expression of KNAT2 and KNAT6 defines the base and boundaries of the SAM (Belles-Boix 2006) respectively. At the same developmental stage, expression of the Class I KNOX transcription factor

KNAT1 delineates the hypocotyl while, at later stages, its expression overlaps with those of STM and KNAT6 (Hay & Tsiantis 2010). While, so far, only one Class II KNOX gene has been described as involved in direction of the SCW development program (KNAT7, (Li et al. 2012)), the Class I KNOX genes KNAT1 and STM play a vital role in the proper formation of secondary xylem, and are therefore of great interest to this thesis.

1.7 Phytohormones

Phytohormones are small molecules produced within the plant which, even at very low concentrations are able to elicit large scale responses in plant development and metabolism. In contrast to animal systems, plants lack specialised hormone-producing tissues (e.g. glands), rather; each plant cell is initially competent to produce and sense hormones. Phytohormones affect all aspects of plant development, from seed germination, to secondary growth, and senescence. Several occurrences of genes encoding the biosynthetic machinery for producing plant hormones have also been described fungi and bacteria. But in these cases, the plant hormones may not fill an endogenous function and are rather, as is the case with auxin in *Agrobacterium tumefasciens* (Nester 2015), used to facilitate the establishment of the inter-organismal relationship. One aspect of particular importance is the ability of hormones to act distally to the cells in which they are produced, i.e. their mobility. This can be achieved through either passive diffusion, or active transport, and modes of transport differ widely between hormones.

There has been a great deal of discussion regarding whether or not the terms hormone, or morphogen (Bhalerao & Bennett 2003; Davies 1995), are applicable in plants, as they were initially borrowed from animal research. For the sake of ease I will henceforth use the terms plant hormone or phytohormone. The known classes of hormones, e.g. auxin, gibberellic acid (GA), jasmonic acid, brassinosteroids and ethylene, have been studied for several decades, but the greatest strides have been made since the early 2000s, primarily owing to the study of Arabidopsis mutants using new, and refined, analysis methods. By now the biosynthetic and signalling pathways are now well understood (Santner et al. 2009).

Basic knowledge of phytohormones and their action was greatly improved during the green revolution, as advances in molecular genetics allowed for directed mutagenesis, and identification of possible traits and related genetic markers. A number of prominent high-yielding varieties of wheat and rice (with impaired GA biosynthesis) were introduced during this time, vastly

outperforming traditional varieties as the plants shifted carbon allocation from stem growth to seeds (Kush & Khush 2001).

The large number of substances which classify as phytohormones are too diverse to cover within the scope of this thesis and thus, I will endeavour to introduce the chief plant hormones involved in xylogenesis; auxin and GA, as well as some other factors affecting cell division and growth prior to xylem cell differentiation.

1.7.1 Auxin

The auxin phytohormone class, of which indole-3-acetic acid (IAA) is the most abundant form, was the first of the major plant hormone classes described by science(Davies 1995). Initially alluded to by Charles Darwin in his experiments investigating differential growth in response to light in 1881, it was later repeated and expanded upon by, Boysen-Jensen (Boysen-Jensen 1913). Building on Darwin's findings, this research indicated that a mobile factor is transported throughout the plant and can affect developmental changes at sites distal to that of their synthesis. This factor was later identified as auxin by Thiman (Thiman 1935). Since then a number of endogenous auxins, along with several synthetic ones, have been identified (Simon & Petrášek 2011; Ludwig-Müller 2011). In spite of this, most research has been focused on understanding the function of IAA. Auxin is one of the main regulators of plant development, involved in directing processes from the very initiation of embryonal growth, to plant senescence (Mueller-Roeber & Balazadeh 2014; Zhao 2010).

Although auxin biosynthesis occurs in both plants and plant-colonising microbes, such as Agrobacterium, this thesis will focus on its biosynthesis and action within plants. Multiple pathways exist through which IAA is produced. Several of these use tryptophan as a starting substrate; however, there is also a tryptophan-independent pathway, which has been reviewed (Zhao 2010).

The main sites of auxin biosynthesis in above-ground tissues are the shoot apex and young leaves. From here auxin is transported to its site of action, either through non-directional transport along with photosynthates in the phloem, or by transporter-mediated export through cells in a polar fashion. This is a slower but more precise means of transport allowing sharp auxin gradients to be established. Partial retention of auxin within the cell is a function of the pK_a (4.7)of auxin, meaning that the cytoplasmic pH of approximately 7 deprotonates IAA (Ljung 2013), effectively trapping it within the cell. In contrast, the auxin pool in the apoplastic space, where pH is two

orders of magnitude lower, becomes partially protonated to IAAH and can then diffuse across the plasma membrane.

The most prominent active form of auxin is believed to be free IAA (Gao & Zhao 2014), and while it's activity is dependent on its carboxyl group, the process of inactivation by oxidation is still not fully understood. Though previous evidence pointed to peroxidases being involved in the process (Normanly 2010), more recent studies have implicated the oxidase proteins DIOXYGENASE FOR AUXIN OXIDATION 1 & 2 (DAO1 &2) (Porco et al. 2016; Mellor et al. 2016) in Arabidopsis. Another means of IAA deactivation is by conjugation into IAA esters, e.g. by conjugation with a sugar, or amino-conjugates (Ludwig-Müller 2011). These IAA esters are suggested to be storage forms (Gao & Zhao 2014) and evidence from Arabidopsis studies have identified the GRETCHEN HAGEN 3 (GH3) family consisting of some 20 amidosynthase genes, as key actors in the formation of IAA-amide conjugates (Hagen et al. 1991). While these conjugates may be released as free IAA upon hydrolysation, indicating they may function as another storage form, others have no known pathway by which they can be hydrolysed, suggesting a role as an auxin-sink (LeClere et al. 2002).

Active auxin elicits transcriptional responses through alteration of the availability of free *AUXIN RESPONSE FACTOR* (*ARF*) encoded proteins. These transcription factors affect gene expression upon homodimerisation and bind to the promoters of auxin-responsive genes. In the absence of auxin, such homodimers are kept from forming by ARF monomers instead heterodimerising with Aux/IAA proteins. Upon perception of auxin by the Skp, Cullin, F-Box containing (SCF) $SCF^{TIR1/AFB1-5}$ complex, the ligase protein contained in the complex affects ubiquitin-mediated proteosomal degradation of the Aux/IAA repressor, allowing transcription of ARF-responsive genes to proceed (Chapman & Estelle 2009).

Aux/IAA factors comprise a large family of proteins, with 29 members identified in Arabidopsis, having largely conserved domain organisation. Of the four domains (DI-IV), DI has been found to interact with the transcriptional repressor TOPLESS (TPL) (Overvoorde et al. 2005) while DII contains a protein stability-modulating 13-amino acid degron motif. Homo- and heterodimerisation are mediated by DIII and IV. Alluded to above, degradation of Aux/IAA proteins in response to auxin is carried out by a family of E3 ubiquitin ligases named SCF, where the F-box protein consists of either a TRANSPORT INHIBITOR RESPONSE1 (TIR1) protein, or a member of the AUXIN SIGNALING F-BOX PROTEIN (AFB), the whole complex denoted $SCF^{TIR1/AFB1-5}$. The F-box protein also directs substrate specificity of the complex, with substrate recognition being dependent on binding of auxin to the

F-Box protein (Kepinski & Leyser 2005; Parry et al. 2009; Dharmasiri et al. 2005). A model of TIR1-Aux/IAA interaction based on structural studies of TIR1 in the presence of auxin and a 13 amino acid degron peptide of Aux/IAA7 suggests that post-translational modifications of TIR1 are not required for Aux/IAA or auxin binding and that binding of auxin does not induce conformational changes in TIR1 (Calderón Villalobos et al. 2012). Instead, presence of auxin in the binding pocket of TIR1 may act as a link, allowing for increased affinity for Aux/IAA interaction partners (Kepinski & Leyser 2005; Parry et al. 2009; Dharmasiri et al. 2005). While all of the TIR1/AFB1-5 proteins have been confirmed as auxin receptors, several studies have indicated that they perform disparate functions *in vivo* (Dharmasiri et al. 2005; Vidal et al. 2010). The presence of so many potential interactors of the Aux/IAA-$SCF^{TIR1/AFB1-5}$ pathway with such individually varying substrate specificities, both for auxin and for interaction partners, indicates that there is a system in place within plants, by which differential auxin sensitivity can be employed to alter gene expression in a hormone level-dependent manner (Calderón Villalobos et al. 2012).

1.7.2 Gibberellic acid

Gibberellic acid was initially identified by Eiichi Kurosawa during studies of rice afflicted by the fungus *Gibberella fujikori* in 1926 and was isolated 12 years later (Yabuta, T. and Sumiki 1938). Gibberellins in plants come in a multitude of forms, but are all diterpenoid acids, produced through the terpenoid synthesis pathway in plastids, and subsequently modified by numerous enzymatic pathways in the ER and cytosol. To date, the enzymes of the GA metabolic pathway, as well as the genes encoding them, have largely been identified and their functions investigated (Yamaguchi 2008). Biosynthesis of the hormone has been suggested to occur mainly in fast-growing tissues (based on data from Arabidopsis, tobacco, and rice) (Silverstone et al. 1997; Aach et al. 1997; Itoh et al. 1999; Kaneko et al. 2003). As these would also be the same location as its sites of action, GA may be synthesised distally and transported as biologically inactive forms to the sites of activity where, by the action of enzymes encoded by the *GIBBERELLIN3 OXIDASE* (GA3ox) gene family, they are metabolised to their bioactive forms. Indeed, while studies (Proebsting et al. 1992; Eriksson 2006; Björklund et al. 2007) indicated that GA is capable of acropetal and basipetal movement, experiments using grafts of Arabidopsis *ga1-3* mutant scions onto wild-type stocks have since shown conclusively that GA is mobile (Ragni et al. 2011). Furthermore, the same study illustrated that this was the case for endogenous

GA, whereas previous investigations had largely relied on exogenous application. Another proposed pathway for this includes transport of the GA precursor ent-kaurene, which can then be released, absorbed, and later metabolised at GA target tissues in Arabidopsis (Otsuka et al. 2004).

Gibberellin is sensed by the receptor protein GA-INSENSITIVE DWARF1(GID1), a soluble protein, which localises to both the nucleus and cytoplasm in rice (Willige et al. 2007; Ueguchi-Tanaka et al. 2005). Though initially identified in rice, studies of the GID1 receptor have revealed a high degree of conservation across plant species (Hauvermale et al. 2012) with at least three homologs (GID1a, b and c) present in Arabidopsis. The function of the GID receptor was initially inferred from studies of mutants, which display a phenotype similar to that of GA biosynthesis mutants, but are unable to be rescued by exogenous GA supplementation. Binding of GA to the GID1 lipase catalytic domain induces conformational changes in the N-terminal domain which enhances the interaction between this domain and the bound GA. This in turn increases the interaction affinity of the GID1-GA complex for DELLA proteins, an interaction which further increases the binding affinity for GA_4, stabilising the whole complex (Nakajima et al. 2006; Ueguchi-Tanaka et al. 2007). DELLA repressors are homologous to metazoan STAT (for Signal Transducers and activators of transcription)-factors and are named for the N-terminal regulatory domain containing a conserved Asp-Glu-Leu-Leu-Ala (DELLA) motif (Hauvermale et al. 2012). Additionally, they also contain a C-terminal GRAS (GAI, RGA, and SCARECROW) domain. These effectors repress gene expression *in planta* by interaction with various target TF proteins, inhibiting them from effecting expression of their intended target genes. Thus, perception of GA may act to both up, or down-regulate gene expression through a single pathway, dependent on the function of each DELLA target (Hauvermale et al. 2012).

The N-terminal domain of DELLA proteins is largely unstructured, however; upon interaction with GID1 it takes on a more ordered form (Sun et al. 2010). Apart from the DELLA motif, it also includes a domain suggested by multiple studies to be involved in protein phosphorylation, with a possible function in DELLA regulation (Hauvermale et al. 2012). DELLA repression is lifted by degradation of DELLA proteins through the ubiquitin-proteasome pathway in response to GA. This was established through studies utilising mutated Arabidopsis SLEEPY (SLY) and rice GID2, both homologous F-box proteins catalysing polyubiquitination of DELLA, which induced GA insensitivity by failing to degrade DELLA proteins (McGinnis 2003; Sasaki Itoh, H. et al. 2003). As with $SCF^{TIR1/AFB1-5}$ mediated degradation of Aux/IAA proteins, the SLY/GID F-box proteins of the $SCF^{SLY2/GID}$-complex confers

substrate specificity through a c-terminal protein-protein interaction domain (Smalle & Vierstra 2004) and the complex is subsequently degraded in a similar manner, allowing gene expression to take place.

1.7.3 Short peptides

In Arabidopsis, the aforementioned factors CLE41 and CLE44 have been shown to be processed into a short peptide signal, known as the tracheary element differentiation inhibitory factor (TDIF), which is perceived by the leucine-rich repeat receptor-like kinase (LRR-RLK), PXY. This signal utilises the transcription factor WUSCHEL HOMEOBOX RELATED 4 (WOX4), to alter downstream gene expression response to the TDIF signal. The 12-amino acid long TDIF peptide is produced from the independent progenitor peptides CLE41 and CLE44, each of which are approximately 100 amino acids in length (Ito 2006). Over-expression of genes from which TDIF is derived has been demonstrated to result in a loss of apical dominance (Strabala 2006) and, in the case of CLE41, 42 or 44, in increased cell division in vascular tissues (Etchells et al. 2015).While this signal transduction system itself may not contribute directly to xylem formation, it is clear from e.g. the *pxy* mutant in Arabidopsis that improper function of this system leads to striking phenotypes in the xylem (Fisher & Turner 2007).

1.8 Hormonal control of xylem differentiation and SCW deposition

A great many hormonal factors contribute to the regulation of wood formation, for a review of the subject I refer to (Sorce et al. 2013). However, to summarise the main concepts; auxin's role in the regulation of cambial activity and differentiation of vessel cells has been extensively studied. Basipetal polar auxin flow, mainly in the vascular cambium (Uggla et al. 1996; Uggla et al. 1998; Sundberg, B., Uggla, C., and Tuominen 2000), ensures continuity of vascular tissues and induces ordered patterning of vascular strands throughout the plant. This flow of auxin is thought to induce cambial divisions as well as the expansion of xylem derivatives. It has also been suggested to trigger the onset of maturation (Sorce et al. 2013), although evidence gained through work for this thesis suggests that the role in maturation is actually the opposite.

Auxin has long been proposed to perform a pivotal role in secondary growth (Sundberg, B., Uggla, C., and Tuominen 2000). Approximately ten years ago, it was proposed to direct key regulators of secondary xylem formation in a concentration-dependent manner (Nilsson et al. 2008), a concept

which has been further studied, and reviewed recently (Bhalerao & Fischer 2014; Bhalerao et al. 2016). The main contribution of auxin to xylogenesis has been previously described as lying in the promotion of cell division activity in the cambium (Nilsson et al. 2008), and the subsequent differentiation and development of vessel cells (Fukuda 2004; Kubo et al. 2005).

Involvement of GA and auxin in xylogenesis has previously been partially studied, for instance; Björklund et al. (2007), investigated the crosstalk between IAA and GA and the effects of depletion by decapitation of these hormones, followed by subsequent supplementation with either hormone, on xylogenesis. That study concluded that GA stimulates polar auxin transport, by induction of Populus PIN-FORMED 1(PIN1) expression, in a synergistic fashion with IAA. The role of GA was further described in the context of fibre elongation and xylogenesis and found to act through homologues of GID1 in Populus (Mauriat & Moritz 2009). This work built on previous findings that measured increased secondary growth and xylem fibre length in plants over-expressing AtGA20ox (Eriksson et al. 2000).

As described earlier, hormone concentrations peak at distinct locales in the cambium and developing xylem. Graded initiation of developmental programs, such as cell expansion and SCW deposition suggests that various tissues may be differentially sensitive to hormones, as discussed previously. Another mode of control of hormone activity, particularly relevant for gibberellic acid, is the transformation between biologically active, and inactive, forms. The connection between hormonal signals and the expression of master level secondary wall TFs affecting fibre cell walls is currently poorly understood. However; this thesis will endeavour to present evidence for just such a connection.

2 Objectives

2.1 General objectives

The aim of this thesis was to investigate the factors controlling SCW formation, specifically in fibre cells, of a commercially important species, and to attempt to identify ways in which this knowledge may be applied for future attempts at engineering biochemical properties of SCWs.

2.2 Specific objectives

To add to the understanding of the connection between plant hormones auxin and gibberellin, and formation of secondary cell walls - building further on knowledge gained through research performed within the bounds of this project, as well as novel findings from the greater scientific community. If possible; apply these findings to develop tools for specific engineering of wood traits by altering the expression of genes responsible for traits of interest.

In context of the papers presented in this thesis:

Paper I: Discuss and suggest a more specific terminology for the field of plant stem cells

Paper II: Establish a method for screening Vessel specific from fibre specific genes by comparison of their expression profiles, protein sequence, and co-expression networks.

Paper III: Attempt to connect the phytohormones auxin and GA directly to the developmental program of secondary xylem fibres.

Paper IV: Investigate the role of GA in regulation of KNOX TF-mediated fibre differentiation.

3 Results and discussion

3.1 Cambial Stem cells and their niche (Paper I)

In this review paper (Paper I), my co-author and I discuss the problems associated with basing the terminology used to describe plant-related phenomena on terminology and concepts borrowed from animal research as there are important differences between plant and animal stem cell systems. We focus our review on the development and differentiation of stem cells in the vascular cambium, and suggest a specific terminology to help avoid confusion when discussing the subject (Paper I, Table 1).

We highlight the need for investigation of cambial tissues in trees, as a complement to studies in Arabidopsis as, even though Arabidopsis hypocotyl is a powerful model system for cambial development; it does not account for the full range of evolved cambial forms. Nor does the rather simplistic genome of Arabidopsis allow for studies of systems in which genome duplications have led to specific neofunctionalisation and redundancies post gene/genome duplication. In this regard, Populus is a more apt model system, although the longer time required for the generation of transgenic lines that can be used for analysis is a major drawback.

3.2 AspWood: High-spatial-resolution transcriptome profiles reveal uncharacterized modularity of wood formation in Populus tremula (Paper II)

In Paper II, I describe the expression patterns of secondary wall NAC domain transcription factors and how those described as master regulators of vessel and fibre differentiation, as well as those directing vessel maturation/PCD, are expressed in 4 distinct domains throughout the studied wood sections.

The early, and rapid, onset of expression observed for *VND6* paralogs centripetally from the zone where cell division occurs, in conjunction with the observation in tangential sections containing radially expanding vessel cells of maximum *VND6* homologue expression (Fig. 1), supports its previously reported role in xylem vessel differentiation. Additionally, *VND6* homologue expression remains throughout the SCW developing zone and exhibits a secondary peak in older cells, which alludes to the possibility of these TFs performing other functions. We suggest that the section with maximum expression of the detected *VND7* homologue and the centripetal peak of the *VND6* paralogues delimits the border between SCW deposition phase and the maturation phase of vessel cells, on the basis of the close correlation between the PCD marker gene *METACASPACE 9* (*MC9*) and *VND7*. Homologues of factors identified as master regulators of fibre differentiation and SCW deposition in fibres, *SND1* and *NST1* are found to be expressed both toward phloem and xylem, indicating that though their function may be specific to fibres, there is no distinction between xylem or phloem fibres. The greatest level of expression of *SND1* and *NST1* homologues was observed centripetally from the cambium, shifted slightly further toward the pith than initiation of *VND6* expression. This correlates closer with the later initiation of xylem fibre SCW biosynthetic genes, which are expressed further centripetally, e.g. in the case of *SND3* homologues and SCW biosynthesis marker gene *CESA8*.

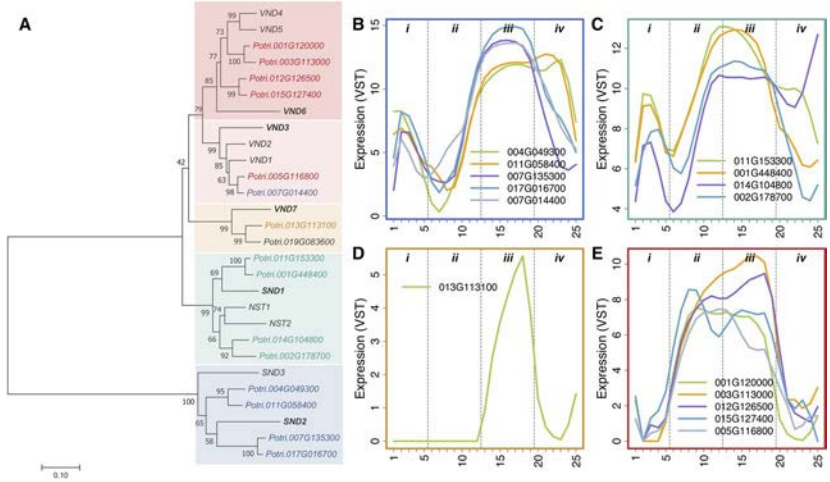

Fig. 1. NAC Domain Transcription Factors Are Expressed in Distinct Patterns Corresponding to Phylogenetic Clustering.

(**A**) Phylogenetic tree of Populus wood associated NAC-domain transcription factors with homologs in Arabidopsis. Colors indicate coexpression. Arabidopsis gene names in bold are used as clade names. (**B**) Expression profiles of Populus *SND2* homologs (Potri.007G135300, Potri.017G016700, Potri.004G049300, and Potri.011G058400) compared with the *VND3* homolog with a similar expression profile (Potri.007G014400). (**C**) Expression profiles of Populus *SND1* homologs (Potri.001G448400, Potri.002G178700, Potri.011G153300, and Potri.014G104800). (**D**) Expression profile of the Populus *VND7* homolog expressed in AspWood (Potri.013G113100; Potri.019G083600 is not expressed). (**E**) Expression profiles of Populus *VND6* homologs (Potri.001G120000, Potri.003G113000, Potri.012G126500, and Potri.015G127400) compared with the *VND3* homolog with a similar expression profile (Potri.005G116800).

My characterisation of SWN expression demonstrate that these occur in distinct zones, which supports functions in direction of differentiation for *SND1*/*NST1*/*VND6* and in the maturation for *SND3*/*VND7* genes. I then tested previously suggested direct targets of the master level transcription factor SND1, identified by either transactivation or Chromatin Immuno-precipitation (ChIP) assays in protoplasts (76 genes), or other more direct lines of evidence (34 genes). This was done by comparison of their expression patterns across wood with that of *SND1* homologue genes. Generation of a co-expression network from these 110 genes yielded a network consisting of 69 nodes, indicating that 41 putative targets were not expressed in wood and, therefore; to be considered as false positives of the previous studies. I found that the ChIP-identified direct targets were particularly spurious, as only 35 of the 76 genes were co-expressed with *SND1* homologues, while 32 of 34 target genes proposed by other methods were included in the network.

The bulk of ChIP-identified targets were found to negatively correlate with the expression of *SND1* homologues, indicating that while their promoters could indeed be direct targets of one, or several *PtrSND1* paralogues; this may result in repression, rather than activation, of these targets. So far, this has not been tested experimentally.

With my contribution to this paper, I show that it is possible to distinguish between master regulators of vessel and fibre cell differentiation and cell wall deposition by virtue of their distinct expression profiles, and I go on to examine previously suggested direct targets of the homologues of one such factor, SND1. This examination revealed that putative targets from earlier studies identified by ChIP were, to a large extent, not expressed in wood, and of the genes that were; many correlate negatively with PtrSND1. This highlights the need for verification of results obtained by methods such as ChIP, as it is prone to the generation of false positives, and also illustrates the power of this method as a tool for screening of potential interactors and targets of transcription factors.

3.3 The plant hormone auxin directs timing of xylem development by inhibition of secondary cell wall deposition through repression of secondary cell wall NAC-domain transcription factors (Paper III)

The motivation behind the study detailed in Paper III was to find a way of affecting selective alteration of cell wall properties in fibre cells, which required identification of a fibre specific promoter. Work in Arabidopsis has identified a group of NAC-domain transcription factors as master regulators of fibre differentiation and development. However, this identification has relied heavily on ectopic overexpression of such factors and therefore required verification.

Using multiple sequence alignments combined with RNAseq data, I investigated a number of candidates, selected from the set of NAC domain TFs expressed in wood, and was able to filter them down by virtue of their phylogeny and expression profiles across Populus wood using a similar approach as established in Paper II.

In this paper, I show that the plant hormone auxin represses the expression of secondary wall master switches on a transcriptional level, and also appears to negatively regulate the formation of secondary cell walls on the anatomical level.

The promoter of *PtrSND1-1* (Potri.011G153300) was selected as the main candidate, and through studying the expression patterns of a 1276 bp and 315 bp fragment of the *PtrSND1-1* promoter by promoter::GUS constructs in Arabidopsis, I found that it matches the expression pattern of the native promoter of the homologous *AtSND1* gene (Fig. 2), though the expression pattern is slightly constrained. Most importantly: the expression is confined to fibre cells and differentiating parts of the cambium, in both stems and hypocotyls of Arabidopsis. I did not observe any expression of GUS in young differentiating vessels, though this may be difficult to score as vessels undergo rapid expansion followed by PCD. I observed the same GUS expression patterns when under the control of either of the isolated promoter fragments, indicating that the cis-elements necessary for direction of expression to fibre cells are present in the short fragment, and that the regulatory machinery appears to be conserved between the two species.

Profiling the cis-elements present in the short promoter revealed a set of 8 interesting elements, of which two in particular stood out; the ARFAT and

GAREAT elements, which are auxin and GA-responsive elements, respectively. The arrangement of these two elements was striking, as ARF-binding elements commonly occur in tandem (Boer et al. 2014). However, this promoter fragment only contains a single copy, and the GAREAT element partially overlaps the region where the second ARFAT element is expected to localise. The exact consequence of this arrangement warrants further study, however, new information gleaned from Paper IV suggests that the GAREAT is very similar to the binding element of a class of transcription factors known as *NUCLEAR FACTOR Y* (*NF-Y*). My initial idea was that the arrangement of these elements could result in binding of a single ARF factor, or an inactive ARF heterodimer, thereby sterically hindering binding of a putative GA-responsive factor. This is merely speculation and will need to be investigated in the future. Irrespective of this, the finding hinted at a signalling mechanism that integrates both auxin and GA signals may have evolved as a means to control the expression of *PtrSND1-1*.

I therefore hypothesised that auxin and GA plays a direct regulatory role in the expression of this gene, and thus, I decided to investigate the effect of hormone treatment on the expression pattern of the short *PtrSND1-1* promoter fragment, and how expression of SWNs would react to the same hormone treatment in native systems. I subjected pPtrSND1-1::GUS Arabidopsis transgenics, as well stem pieces of wild type Arabidopsis and tissue-cultured Populus to either auxin (NAA) or GA (GA3) for 4 hours, and also performed a long-term treatment of green-house grown Populus plants. I found that short term GA treatment of Arabidopsis inflorescence stems expands the GUS expression pattern to appear more like that of native *pAtSND1* (Fig. 2), while NAA treatment appears to slightly restrict expression.

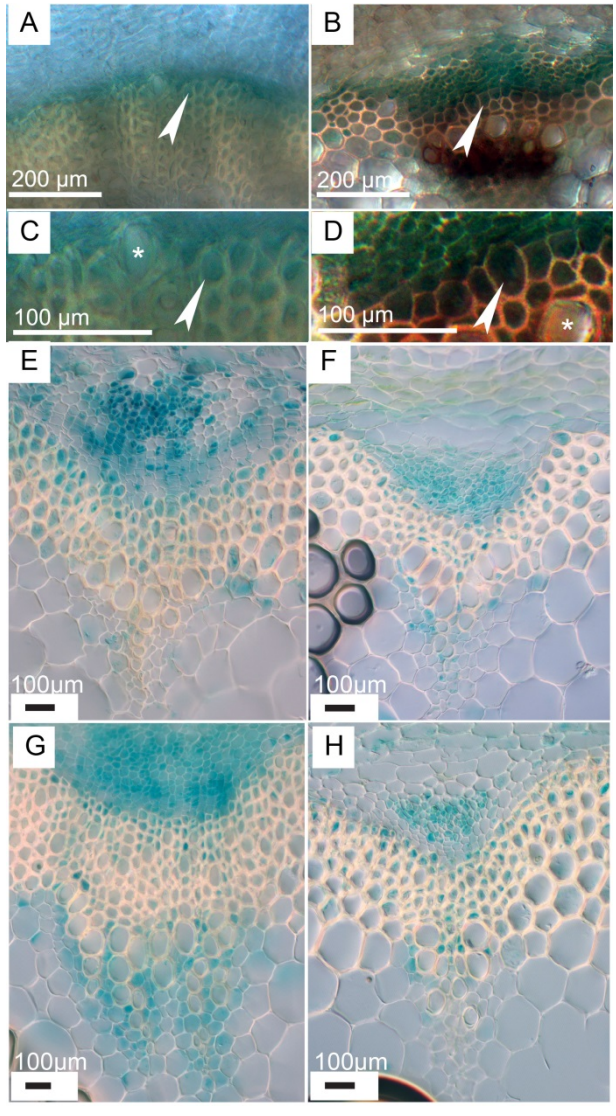

Fig. 2. GUS signal from plants transformed with β-glucuronidase (GUS) under the control of a 315bp promoter fragment of PttSND1-1. **A, B**. GUS signal in the hypocotyl of 30 day-old Arabidopsis. Signal is visible in cells directly adjacent to, and inside fibre cells undergoing SCW deposition (Arrow), but not in vessel cells (*). **C, D**. GUS signal in the inflorescence stems of 30-day old Arabidopsis. Signal is visible in cambial cells close to interfascicular (IF) fibres (Arrow), as well as in adjacent fibre cells undergoing SCW deposition. No signal is visible in vessel cells. **E**. Expression pattern of pAtSND1, untreated control. **F**. Expression pattern of 315bp pPtrSND1-1 after 4h DMSO treatment. **G**. Expression pattern of 315bp pPtrSND1-1 after 4h GA treatment. **H.** Expression pattern of 315bp pPtrSND1-1 after 4h NAA treatment.

Quantification of gene expression in response to hormone treatments by qRTPCR showed that SND1 orthologues tend to be down-regulated by NAA in Populus, as is one of the studied NST1 homologues. GA treatment has a general inductive effect on all tested genes (Paper III, Fig. 5). The inductive effect of GA is also observed in Arabidopsis, however; I did not observe the same effect upon auxin-treatment. Whether this discrepancy is due to an actual difference in expression or to the fact that fibres constitute a much smaller part of the whole tissue sampled, leading to a dilution of mRNA abundance, is still unclear. Long term treatment of decapitated Populus stems showed that GA is able to rescue cell division and expansion to a greater extent than NAA (Fig. 3), but leads to an initial deficiency in vessel formation, likely caused by the loss of apical auxin.

Secondly, while GA supplementation lead to an almost wild type phenotype in the cell walls of fibres, NAA treated plants have significantly thinner cell walls (Fig. 3), which also appear less lignified than in the controls, or GA-treated, plants.

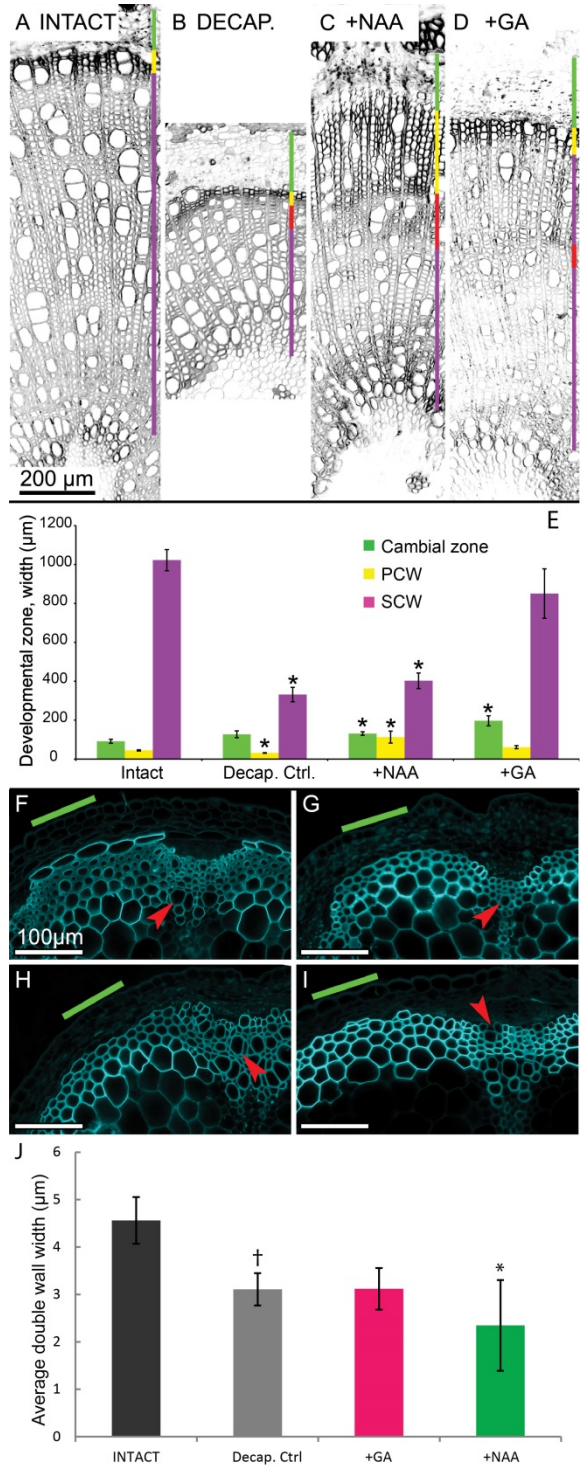
A INTACT
B DECAP.
C +NAA
D +GA
200 µm
E
Developmental zone, width (µm)
1200
1000
800
600
400
200
0
Cambial zone
PCW
SCW
Intact
Decap. Ctrl.
+NAA
+GA
F
G
100µm
H
I
J
Average double wall width (µm)
6
5
4
3
2
1
0
INTACT
Decap. Ctrl
+GA
+NAA

Fig. 3. Effects of NAA or GA-treatment on Populus and Arabidopsis anatomy. **A-D**: Coloured vertical bars denote developmental zones Green=cambium, Yellow= non-lignified cell wall zone, Purple= secondary cell wall deposition and cell maturation. Red bar denotes transition zones where a temporary lack of apically supplied hormones, due to decapitation has led to late-wood like anatomy. **E**. Widths of developmental zones under NAA or GA treatment. Changes in developmental zone width (µm) and the effect of NAA or GA treatment in cambium (green), non-lignified cell wall zone (yellow), secondary cell wall deposition/maturation zone (magenta). Stars denote significant difference versus zone width in intact controls (P>=0.05, N=5), bars denote standard error. **F-I**: Calcofluor white stained transverse sections of Arabidopsis inflorescence stems. **F**: Intact, **G**: Decapitated DMSO treated, **H**: Decapitated GA-treated, **I**: Decapitated NAA-treated. **J**: Cell wall thickness in respective treatment. † denotes significant difference compared to intact control. * denotes significant difference compared to decapitated, DMSO-treated control.

Though there is likely a great deal of interplay between the GA and auxin pathways in the tissues studied in Paper III which needs to be further clarified; it is clear that auxin appears to have an, if not repressive, then at least a delaying effect on SCW formation in the secondary xylem of Populus. I speculate that this is due to an evolutionary pressure for avoiding vessel expansion in a micro-environment where fibres are developing SCWs in parallel. Such a case would likely restrict the ability of vessels to expand, negatively impacting water transport capacity. A likely example of this can be seen in auxin insensitive plants, such as *axr2-1* mutant lines, or indeed in over-expressor of *SND1* plants, which both form ectopic secondary cell walls and display severely dwarfed phenotypes.

The somewhat inconsistent findings of hormone treatments in Populus and Arabidopsis encouraged me to investigate Populus plants with altered endogenous hormone transport or biosynthesis.

A line over-expressing the auxin transport protein PIN-FORMED 5 (PIN5) was studied, as these plants sequester auxin inside the cells at the site of biosynthesis, leading to a lack of the hormone available for transport throughout the plant. These plants displayed ectopic formation of xylem and phloem fibres (Paper III, fig 6, D, F), along with severely stunted growth. This is possibly caused by a lower incidence of vessel differentiation that also fails to properly expand, likely due to restrictive surrounding cell walls.

The effect of altered GA availability was studied in GA20ox over-expressor plants, producing more bioactive GA. In these plants, we found that there was a tendency for SWNs to be induced similar to the GA-treated Populus stems.

The alteration of expression patterns in response to hormone treatment, prompted me to query the AspWood database (established in Paper II) to investigate if the direct co-expression neighbourhoods of the Populus

secondary wall NAC master switches contain any genes related to auxin or GA-biosynthesis, signalling or reception. What I found was a diverse number of Aux/IAA transcriptional repressor-encoding genes (Paper III, fig 4), co-expressed mainly with the various *PtrVND* homologues. The *PtrVND* homologues expressed closest to the cambium are also co-expressed with the GA transporter-encoding gene *NPF3*, which is involved in GA homeostasis in Arabidopsis. In contrast, *VND* genes proposed to be involved in vessel cell maturation and PCD co-express with a separate set of Aux/IAA genes, and a homologue of *RGA*. Homologues of *SND1* and *NST1* co-vary positively with the GA-receptor homolog *GID1B*, and with one *Aux/IAA* gene.

Co-expression of a number of *Aux/IAA* genes with PtrSWNs hints at a regulatory machinery able to differentially sense auxin concentrations. The actuality of such a regulatory system still needs to be studied but it would provide an explanation for how plants are able to make use of previously described differential phytohormone concentrations across wood forming tissues (Immanen et al. 2016). It is possible that the specific *Aux/IAA* genes co-expressed with VND orthologues in Populus have auxin affinities that allow them to repress expression of *SND1* and *NST1* orthologues. However, this is speculation and will have to be experimentally verified in the future.

A closer look at the co-expression neighbourhoods of *PtrSND1-1*, *PtrSND1-2*, *PtrSND2-2*, *PtrVND6-3* and *PtrVND7-1* revealed that there is overlap within their phylogenetic clades, but not between them. The neighbourhood of higher order TFs, e.g. *PtrSND1-1*, are enriched in other TFs while the neighbourhoods of their downstream targets, e.g. *PtrSND2-1*, contain mainly cell wall biosynthetic genes (Paper III, Supplemental Figure 2). In conclusion, differentiation of secondary xylem is an incredibly complex developmental program, that is likely under the control of a signalling cascade and begins with the integrated sensing of GA and auxin, and is possibly able to distinguish different hormone concentrations (at least in the case of auxin). The presence of an elevated auxin concentration has been shown to promote differentiation, and development, of xylem vessels cells, but in this Paper I show that auxin also acts to delay the deposition and development of SCWs in non-vessel cells.

3.4 Class I KNOX transcription factors interact with the NF-Y complex to promote fibre differentiation in the hypocotyl

This paper sought to elucidate the functional difference of class I KNOX TFs in secondary versus primary growth, as described by Liebsch et al. (2014). A suggested explanation is that differential sets of interaction partners are present in the two meristems. We investigated this by a yeast-2-hybrid screen. Utilising full-length STM and KNAT1 proteins, in contrast to previous Y2H screens, we identified 15 and 22 putative candidates for KNAT1 and STM respectively. Both bait-proteins interacted with a member of the NUCLEAR FACTOR-Y (NF-Y) protein family, specifically the C-subunit of NF-YC3 and NF-YC9. To ensure that the observed interactions were biologically relevant, a cDNA library constructed from several different tissues and organs at various stages of development, including both primary and secondary meristems was used.

The NF-Y-complex (a trimer consisting of subunits A, B, and C) regulates gene expression in a wide range of processes (Zhao et al. 2017) depending on the subunit composition. Apical tissues only exhibit expression of *NF-YC* at the site of the incipient leaf and, thus, expression does not overlap with that of either *KNAT1* or *STM*. On the contrary, observed overlap in secondary tissues of the hypocotyl supports the putative interaction between NF-YC and homeobox transcription factors. Such an interaction may play a part in regulating the differentiation of meristematic stem cells to xylem.

Analysis of first order mutants of *NF-YC* genes yielded no striking phenotype, though the high level of redundancy expected in the NF-YC family may be an explanation for this. As fibre formation or plant architecture in these mutants was not affected, we instead chose to focus on studying mutants of other known interactors of NF-YC, such as DELLAs, REF6, CO, and BRM. We were able to show that a mutant of the latter, and a *DELLA* quintuple mutant stabilised DELLA, which displayed phenotypes similar to *knat1* and *stm* mutants.

DELLA interaction with NF-Y was of particular interest as it could act as a competitor for KNAT1 in the absence of GA. The ability of stabilised DELLA to phenocopy *knat1* suggests direct connection of GA to the regulation of secondary xylem formation, which is further supported by evidence of *knat1* mutants being insensitive to GA (Ikematsu et al. 2016). Our replication confirmed this insensitivity in *knat1* mutants, but also demonstrated that increased GA, either by exogenous application, or through over-expression of

GA20ox, increased the ratio of xylem II / xylem I (Paper IV, Fig. 4). Additionally, a *ga3ox* double mutant displayed almost complete loss of fibres, solidifying the idea that GA directs fibre formation through KNAT1.

Transcript quantification of GA biosynthesis genes in the *knat1* mutant found a sharp decrease in *GA20ox* and *GA3ox* transcripts, while *GA2ox2*, involved in GA catabolism was over-expressed (Paper IV, Fig. 5). We confirmed this by investigation of a KNAT1-GR fusion construct under the control of the *KNAT1* promoter in a knat1-null mutant background. Upon dexamethasone treatment we found that the transcription of *GA20ox* was significantly increased, while transcript levels of *KNAT1* were significantly decreased, hinting at negative feedback regulation. While class I KNOX TFs are suggested to negatively regulate GA biosynthesis in the shoot apex, our findings support a model for the vascular cambium where GA acts upstream of KNAT1; resulting in positive feed forward regulation by activated expression of GA biosynthetic genes.

In conclusion: in Paper IV we present an updated model for how KNAT1 interacts with NF-YC in the presence of GA to repress transcription of *BOP1*, thereby promoting fibre differentiation in the secondary hypocotyl. As part of this model we describe a positive feedback regulation of this system by induction of GA biosynthetic genes. Our model suggests that the distinct meristematic function of KNAT1 and STM may be reliant on the presence or absence of NF-YC interaction partners for these proteins.

4 Conclusions and future perspectives

From my research, I established that auxin is a negative regulator of SCW formation in non-vessel cells (Paper III). I also observed that GA promotes fibre differentiation in the secondary hypocotyl of Arabidopsis (Paper IV), through the interaction Class I Homeobox TF KNAT1 with an NF-Y complex. Recently, I have also found that the binding site of NF-Y may be present in the promoter of secondary wall master regulator *PtrSND1-1* (described in Paper III). These observations are in accord with each other, with the distinct expression domains of secondary wall master switches specific to vessels and fibres, described in Paper II, and the distribution of hormone concentration peaks published previously (Immanen et al. 2016). Together, this evidence paints a picture of the hormonal regulation of fibre differentiation and secondary cell wall formation in wood forming tissues. Namely; the presence of high auxin concentration in the cambial niche promotes differentiation of xylem vessels while at the same time repressing the SCW biosynthetic program in surrounding non-vessel cells. As auxin concentration decreases, this repression is lifted. It is unclear whether auxin has a repressive effect on *KNAT1* and *STM* expression in the hypocotyl, though it has been described in leaf primordia (Hay et al. 2006) and, if present, would act in concert with the SCW repression, by repressing fibre differentiation. The shift in auxin:GA ratio allows for de-repression, and subsequent induction, of transcription of the SCW master switches in fibre (non-vessel) cells.

Such a model would make sense from an evolutionary standpoint, as this control mechanism allows plants to form properly expanded vessels, thereby optimising water transport capacity per unit area of the trunk. This allows for the plant to instead focus its energy and resources towards lateral growth and defence against pathogens and pests.

Though the exact regulatory mechanism controlling expression of *PtrSND1-1* is still unknown, it is possible that transcriptional activation occurs

as a result of a KNAT1/NF-Y complex binding to this site. This is particularly notable in light of the recent finding that the ARFAT motif in the short promoter fragment isolated from pPtrSND1-1 overlaps with a partial NFYBE motif (data not shown) on the opposite strand,

There are several lines of inquiry that I would suggest to further our knowledge of this field. Firstly;

It would be very interesting to investigate whether or not the KNOX/NF-Y complex can bind to pPtrSND1-1 and, additionally, if such binding is affected by the presence of auxin. Though such an experiment may be difficult to perform *in vivo*, cell-free systems may make it possible. Similarly, mutagenesis of the GAREAT and ARFAT elements could be carried out, utilising novel CRISPR-based techniques, and the resulting phenotype studied. Such an experiment was initiated as part of this thesis, however; it was abandoned due to time constraints.

Secondly, if either of these elements are found to result in a silencing of gene expression in the presence of auxin, I would suggest the creation of a synthetic promoter incorporating this element, or a corresponding element with the same functionality, in a strong promoter such as CAMV 35S. If functional, this could lead to a way around the problems with ubiquitous cell wall alteration described in previous research.

Aside from the points discussed above, it would be very interesting to characterise the Aux/IAA proteins found to co-express with SWNs in Paper III. While this could initially be done by bioinformatic methods, functional characterisation of, for example, their individual auxin affinities would be crucial. This would allow us to better explain how plants sense and respond to varying auxin concentrations. The power of using large scale RNA-sequencing data is well described in Paper II. One key study for furthering our understanding of the influence of phytohormones on wood formation would be to investigate the expression of these genes across the lateral axis of the plant, i.e. to perform multiple RNA-sequencing series (such as the one in Paper II), but distributed across the vertical length of the plant. This would allow us to better discern the transition from primary to secondary growth, and the factors involved in subsequent maturation stages. Additionally, complementary measurements of the distribution of phytohormonal concentrations in the same samples would be highly informative.

Continued study of the NF-Y and KNOX interaction is also essential. It would be interesting to mis-express the KNAT1 and STM interacting subunits

in the apical meristems, particularly the SAM. I hypothesise that this could lead to a phenocopy of *stm* and *knat1*, in which the meristematic cells differentiate, leading to a loss of the meristem and cessation of primary growth. Due to the potentially early-lethal phenotype of such transgenic plants, utilisation of our dexamethasone-inducible system (used in Paper IV) would be advantageous.

A study of direct interactors of the SND and NST proteins would also be in order. I am not aware of any such data being publicly available, but it could point to new regulatory pathways directing wood formation.

To conclude: Future investigations should be focused on defining and optimising applications of these findings towards novel scientific and industrial purposes. I believe the potential of genetically altered trees to benefit the environment, through carbon sequestration and improvement of industrial processes, is immense. Continued work to facilitate this is crucial, both of biotechnologies and the legal regulatory framework underpinning their use.

The findings described in this thesis, and elsewhere in literature, imply that what is commonly known as “fibre cell fate” is not necessarily defined by internal lineage factors. It may rather be the “default state” of xylem cells, with vessel cells presenting a special, developmentally privileged, case. This would imply that previous strategies for altering wood characteristics by genetic engineering may need to be re-evaluated. The common notion of over-expressing genes encoding gainful enzymes exclusively in fibres should be revised to focus on directing expression to non-vessel cells. Hopefully, the data presented in this thesis, particularly the implication of auxin as a repressive signal in SCW formation, will be of benefit to the future development of such a system.

5 References

Aach, H. et al., 1997. ent-Kaurene synthase is located in proplastids of meristematic shoot tissues. *Planta*, 202(2), pp.211–219.

Alejandro, S. et al., 2012. AtABCG29 is a monolignol transporter involved in lignin biosynthesis. *Current Biology*, 22(13), pp.1207–1212. Available at: http://dx.doi.org/10.1016/j.cub.2012.04.064.

Allen, R.S. et al., 2007. Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis miR159 family. *Proceedings of the National Academy of Sciences*, 104(41), pp.16371–16376. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.0707653104.

Araki, S. et al., 2004. Mitotic cyclins stimulate the activity of c-Myb-like factors for transactivation of G2/M phase-specific genes in tobacco. *Journal of Biological Chemistry*, 279(31), pp.32979–32988.

Architecture, P., 2017. Oakwood Timber Tower, London, UK. Available at: http://www.plparchitecture.com/oakwood-timber-tower.html [Accessed March 15, 2018].

Belles-Boix, E., 2006. KNAT6: An Arabidopsis Homeobox Gene Involved in Meristem Activity and Organ Separation. *the Plant Cell Online*, 18(8), pp.1900–1907. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.106.041988.

Berthet, S. et al., 2011. Disruption of LACCASE4 and 17 Results in Tissue-Specific Alterations to Lignification of Arabidopsis thaliana Stems. *The Plant Cell*, 23(3), pp.1124–1137. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.110.082792.

Bhalerao, R.P. & Bennett, M.J., 2003. The case for morphogens in plants. *Nature Cell Biology*, 5(11), pp.939–943.

Bhalerao, R.P. & Fischer, U., 2014. Auxin gradients across wood - instructive or incidental? *Physiologia Plantarum*, 151(1), pp.43–51. Available at: http://doi.wiley.com/10.1111/ppl.12134.

Bhalerao, R.P., Fischer, U. & Turner, S., 2016. Environmental and hormonal control of cambial stem cell dynamics. *Journal of Experimental Botany*, 68(1), pp.79–87.

Björklund, S. et al., 2007. Cross-talk between gibberellin and auxin in development

of Populus wood: Gibberellin stimulates polar auxin transport and has a common transcriptome with auxin. *Plant Journal*, 52(3), pp.499–511.
Boer, D.R. et al., 2014. Structural basis for DNA binding specificity by the auxin-dependent ARF transcription factors. *Cell*, 156(3), pp.577–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24485461 [Accessed July 14, 2014].
Bonawitz, N.D. & Chapple, C., 2013. Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Current Opinion in Biotechnology*, 24(2), pp.336–343. Available at: http://dx.doi.org/10.1016/j.copbio.2012.11.004.
Bonawitz, N.D. & Chapple, C., 2010. The Genetics of Lignin Biosynthesis: Connecting Genotype to Phenotype. *Annual Review of Genetics*, 44(1), pp.337–363. Available at: http://www.annualreviews.org/doi/10.1146/annurev-genet-102209-163508.
Boysen-Jensen, P., 1913. Über die Leitung des phototropischen Reizes in der Avenakoleoptile. *Berichte der Deutschen Botanischen Gesellschaft*, 31, pp.559–566.
Brackmann, K. & Greb, T., 2014. Long- and short-distance signaling in the regulation of lateral plant growth. *Physiologia Plantarum*, 151(2), pp.134–141. Available at: http://doi.wiley.com/10.1111/ppl.12103.
Bringmann, M. et al., 2012. POM-POM2/CELLULOSE SYNTHASE INTERACTING1 Is Essential for the Functional Association of Cellulose Synthase and Microtubules in Arabidopsis. *The Plant Cell*, 24(1), pp.163–177. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.111.093575.
Calderón Villalobos, L.I.A. et al., 2012. A combinatorial TIR1/AFB–Aux/IAA co-receptor system for differential sensing of auxin. *Nature Chemical Biology*, 8(5), pp.477–485. Available at: http://www.nature.com/doifinder/10.1038/nchembio.926.
Chapman, E.J. & Estelle, M., 2009. Mechanism of Auxin-Regulated Gene Expression in Plants. *Annual Review of Genetics*, 43(1), pp.265–285. Available at: http://www.annualreviews.org/doi/10.1146/annurev-genet-102108-134148.
Chen, S., Lewallen, M. & Xie, T., 2013. Adhesion in the stem cell niche: biological roles and regulation. *Development (Cambridge, England)*, 140(2), pp.255–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3597204&tool=pmcentrez&rendertype=abstract.
Christensen-Dalsgaard, K.K. & Tyree, M.T., 2014. Frost fatigue and spring recovery of xylem vessels in three diffuse-porous trees in situ. *Plant, Cell and Environment*, 37(5), pp.1074–1085.
Condic, M.L., 2014. Totipotency: What It Is and What It Is Not. *Stem Cells and Development*, 23(8), pp.796–812. Available at: http://search.ebscohost.com/login.aspx?direct=true&db=buh&AN=15996180&site=ehost-live.
Creux, N.M. et al., 2013. Induced somatic sector analysis of cellulose synthase (CesA) promoter regions in woody stem tissues. *Planta*, 237(3), pp.799–812.

Davies, P.J., 1995. *Plant Hormones* P. J. Davies, ed., Dordrecht: Springer Netherlands. Available at: http://link.springer.com/10.1007/978-94-011-0473-9.

Dharmasiri, N., Dharmasiri, S. & Estelle, M., 2005. The F-box protein TIR1 is an auxin receptor. *Nature*, 435(7041), pp.441–445. Available at: http://www.nature.com/articles/nature03543.

Djerbi, S. et al., 2005. Carbohydrate-active enzyme involved in the secondary cell wall biogenesis in hybrid aspen. *Plant Physiology*, 137(March), pp.983–997.

Dubos, C. et al., 2010. MYB transcription factors in Arabidopsis. *Trends in Plant Science*, 15(10), pp.573–581.

Edwards, D., Davies, K.L. & Axe, L., 1992. A vascular conducting strand in the early land plant Cooksonia. *Nature*, 357(6380), pp.683–685. Available at: http://dx.doi.org/10.1038/357683a0.

Endrizzi, K. et al., 1996. The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. *The Plant journal : for cell and molecular biology*, 10(6), pp.967–79. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9011081.

Eriksson, M.E. et al., 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology*, 18(7), pp.784–788. Available at: http://www.nature.com/doifinder/10.1038/77355.

Eriksson, S., 2006. GA4 Is the Active Gibberellin in the Regulation of LEAFY Transcription and Arabidopsis Floral Initiation. *the Plant Cell Online*, 18(9), pp.2172–2181. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.106.042317.

Esau, K. et al., 2009. *Esaus Pflanzenanatomie*, Berlin, New York: Walter de Gruyter. Available at: https://www.degruyter.com/view/books/9783110211320/9783110211320/9783110211320.xml.

Etchells, J.P. et al., 2015. A brief history of the TDIF-PXY signalling module: balancing meristem identity and differentiation during vascular development. *New Phytologist*.

Etchells, J.P. & Turner, S.R., 2010. The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. *Development (Cambridge, England)*, 137(5), pp.767–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20147378.

Eudes, A. et al., 2014. Lignin bioengineering. *Current opinion in biotechnology*, 26, pp.189–98. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24607805 [Accessed October 3, 2014].

Fàbregas, N. et al., 2015. Auxin Influx Carriers Control Vascular Patterning and Xylem Differentiation in Arabidopsis thaliana. *PLoS Genetics*, 11(4), pp.1–26.

Fagerstedt, K. V. et al., 2010. Cell wall lignin is polymerised by class III secretable

plant peroxidases in Norway spruce. *Journal of Integrative Plant Biology*, 52(2), pp.186–194.
Fernandes, A.N. et al., 2011. Nanostructure of cellulose microfibrils in spruce wood. *Proceedings of the National Academy of Sciences*, 108(47), pp.E1195–E1203. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1108942108.
Fisher, K. & Turner, S., 2007. PXY, a Receptor-like Kinase Essential for Maintaining Polarity during Plant Vascular-Tissue Development. *Current Biology*, 17(12), pp.1061–1066.
Floudas, D. et al., 2012. The Paleozoic Origin of Enzymatic Lignin Decomposition Reconstructed from 31 Fungal Genomes. *Science*, 336(6089), pp.1715–1719. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1221748.
Freudenberg, K., 1959. Biosynthesis and constitution of lignin. *Nature*, (183), pp.1152–1155.
FSC, 2015. Application of Suzano for commercial use of genetically modified trees. *FSC Worldwide*. Available at: https://ic.fsc.org/en/news-updates/id/1121 [Accessed March 15, 2018].
Fukuda, H., 2004. Signals that control plant vascular cell differentiation. *Nature reviews. Molecular cell biology*, 5(5), pp.379–391. Available at: http://www.nature.com/doifinder/10.1038/nrm1364.
Gallois, J.-L. et al., 2002. Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in Arabidopsis. *Development (Cambridge, England)*, 129(13), pp.3207–17. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12070095.
Gao, Y. & Zhao, Y., 2014. Auxin and Its Role in Plant Development. , pp.21–39. Available at: http://link.springer.com/10.1007/978-3-7091-1526-8.
Gruel, J. et al., 2016. An epidermis-driven mechanism positions and scales stem cell niches in plants. *Science advances*, 2(1), p.e1500989.
Hagen, G. et al., 1991. Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. *Plant Molecular Biology*, 17(3), pp.567–579.
Hauvermale, a. L., Ariizumi, T. & Steber, C.M., 2012. Gibberellin Signaling: A Theme and Variations on DELLA Repression. *Plant Physiology*, 160(1), pp.83–92.
Hay, A., Barkoulas, M. & Tsiantis, M., 2006. ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in Arabidopsis. *Development*, 133(20), pp.3955–3961. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16971475.
Hay, A. & Tsiantis, M., 2010. KNOX genes: versatile regulators of plant development and diversity. *Development*, 137(19), pp.3153–3165. Available at: http://dev.biologists.org/cgi/doi/10.1242/dev.030049.
Hirakawa, Y. et al., 2008. Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proceedings of the National Academy of Sciences of the United States of America*, 105(39), pp.15208–13. Available

at: http://www.ncbi.nlm.nih.gov/pubmed/18812507.
Hu, R. et al., 2010. Comprehensive Analysis of NAC Domain Transcription Factor Gene Family in Populus trichocarpa. *BMC Plant Biology*, 10(1), p.145. Available at: http://bmcplantbiol.biomedcentral.com/articles/10.1186/1471-2229-10-145.
Hussey, S.G. et al., 2011. SND2, a NAC transcription factor gene, regulates genes involved in secondary cell wall development in Arabidopsis fibres and increases fibre cell area in Eucalyptus. *BMC plant biology*, 11(1), p.173. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3289092&tool=pmcentrez&rendertype=abstract [Accessed June 20, 2013].
Ikematsu, S. et al., 2016. ERECTA -family receptor kinase genes redundantly prevent premature progression of secondary growth in the Arabidopsis hypocotyl. *New Phytologist*, pp.1697–1709. Available at: http://doi.wiley.com/10.1111/nph.14335.
Ikeuchi, M. et al., 2016. Plant regeneration: cellular origins and molecular mechanisms. *Development*, 143(9), pp.1442–1451. Available at: http://dev.biologists.org/lookup/doi/10.1242/dev.134668.
Immanen, J. et al., 2016. Cytokinin and Auxin Display Distinct but Interconnected Distribution and Signaling Profiles to Stimulate Cambial Activity. *Current Biology*, 26(15), pp.1990–1997.
Ito, Y., 2006. Dodeca-CLE Peptides as Suppressors of Plant Stem Cell Differentiation. *Science*, 313(5788), pp.842–845. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1128436.
Itoh, H. et al., 1999. The gene encoding tobacco gibberellin 3β-hydroxylase is expressed at the site of GA action during stem elongation and flower organ development. *Plant Journal*, 20(1), pp.15–24.
Jacobs, A. & Dahlman, O., 2001. Characterization of the molar masses of hemicelluloses from wood and pulps employing size exclusion chromatography and matrix-assisted laser desorption lonization time-of-flight mass spectrometry. *Biomacromolecules*, 2(3), pp.894–905.
Johnsson, C. & Fischer, U., 2016. Cambial stem cells and their niche. *Plant Science*, 252, pp.239–245. Available at: http://dx.doi.org/10.1016/j.plantsci.2016.08.002.
Kaneda, M. et al., 2008. Tracking Monolignols during Wood Development in Lodgepole Pine. *Plant Physiology*, 147(4), pp.1750–1760. Available at: http://www.plantphysiol.org/cgi/doi/10.1104/pp.108.121533.
Kaneko, M. et al., 2003. Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? *Plant Journal*, 35(1), pp.104–115.
Kenrick, P. et al., 2012. A timeline for terrestrialization: consequences for the carbon cycle in the Palaeozoic. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1588), pp.519–536. Available at: http://rstb.royalsocietypublishing.org/cgi/doi/10.1098/rstb.2011.0271.
Kepinski, S. & Leyser, O., 2005. The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature*, 435(7041), pp.446–451.

Klarin, G., 2015. Genmodifiering med ny teknik kan ske utan tillstånd. *Vetenskapsradion, SR*. Available at: Genmodifiering med ny teknik kan ske utan tillstånd [Accessed March 15, 2018].
Klemm, D. et al., 2005. Cellulose: Fascinating biopolymer and sustainable raw material. *Angewandte Chemie - International Edition*, 44(22), pp.3358–3393.
Kubo, M. et al., 2005. Transcription switches for protoxylem and metaxylem vessel formation. *Genes and Development*, 19(16), pp.1855–1860.
Kush, G.S. & Khush, G.S., 2001. Green revolution: the way forward. *Nature Reviews Genetics*, 2(10), pp.815–822. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11584298.
Laimer, M. & Rücker, W., 2003. *Plant Tissue Culture* M. Laimer & W. Rücker, eds., Vienna: Springer Vienna. Available at: http://link.springer.com/10.1007/978-3-7091-6040-4.
Lairson, L.L. et al., 2008. Glycosyltransferases: Structures, Functions, and Mechanisms. *Annual Review of Biochemistry*, 77(1), pp.521–555. Available at: http://www.annualreviews.org/doi/10.1146/annurev.biochem.76.061005.092322.
LeClere, S. et al., 2002. Characterization of a family of IAA-amino acid conjugate hydrolases from Arabidopsis. *Journal of Biological Chemistry*, 277(23), pp.20446–20452.
Lee, C. et al., 2009a. Down-regulation of PoGT47C expression in poplar results in a reduced glucuronoxylan content and an increased wood digestibility by cellulase. *Plant and Cell Physiology*, 50(6), pp.1075–1089.
Lee, C. et al., 2009b. The poplar GT8E and GT8F glycosyltransferases are functional orthologs of arabidopsis parvus involved in glucuronoxylan biosynthesis. *Plant and Cell Physiology*, 50(11), pp.1982–1987.
Lenhard, M., Jürgens, G. & Laux, T., 2002. The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. *Development (Cambridge, England)*, 129(13), pp.3195–206. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12070094.
Li, E. et al., 2012. The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in Arabidopsis and is functionally conserved in Populus. *New Phytologist*, 7(1), pp.102–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22236040 [Accessed April 18, 2013].
Liebsch, D. et al., 2014. Class I KNOX transcription factors promote differentiation of cambial derivatives into xylem fibers in the Arabidopsis hypocotyl. *Development*, 141, pp.4311–4319. Available at: http://dev.biologists.org/cgi/doi/10.1242/dev.111369.
Liu, T. et al., 2014. Genome-Wide Analysis and Expression Patterns of NAC Transcription Factor Family Under Different Developmental Stages and Abiotic Stresses in Chinese Cabbage. *Plant Molecular Biology Reporter*, 32(5), pp.1041–1056.
Ljung, K., 2013. Auxin metabolism and homeostasis during plant development.

Development, 140(5), pp.943–950. Available at: http://dev.biologists.org/cgi/doi/10.1242/dev.086363.
Long, J.A. et al., 1996. A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature*, 379(6560), pp.66–69.
Ludwig-Müller, J., 2011. Auxin conjugates: their role for plant development and in the evolution of land plants. *Journal of Experimental Botany*, 62(6), pp.1757–1773. Available at: https://academic.oup.com/jxb/article-lookup/doi/10.1093/jxb/erq412.
Mauriat, M. & Moritz, T., 2009. Analyses of GA20ox- and GID1-over-expressing aspen suggest that gibberellins play two distinct roles in wood formation. *Plant Journal*, 58(6), pp.989–1003.
McFarlane, H.E., Döring, A. & Persson, S., 2014. The Cell Biology of Cellulose Synthesis. *Annual Review of Plant Biology*, 65(1), pp.69–94. Available at: http://www.annualreviews.org/doi/10.1146/annurev-arplant-050213-040240.
McGinnis, K.M., 2003. The Arabidopsis SLEEPY1 Gene Encodes a Putative F-Box Subunit of an SCF E3 Ubiquitin Ligase. *THE PLANT CELL ONLINE*, 15(5), pp.1120–1130. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.010827.
Mellor, N. et al., 2016. Dynamic regulation of auxin oxidase and conjugating enzymes *AtDAO1* and *GH3* modulates auxin homeostasis. *Proceedings of the National Academy of Sciences*, 113(39), pp.11022–11027. Available at: http://www.pnas.org/lookup/doi/10.1073/pnas.1604458113.
Morgan, J.L.W., Strumillo, J. & Zimmer, J., 2012. Crystallographic snapshot of cellulose synthesis and membrane translocation. *Nature*, 493(7431), pp.181–186. Available at: http://www.nature.com/doifinder/10.1038/nature11744.
Mueller-Roeber, B. & Balazadeh, S., 2014. Auxin and Its Role in Plant Senescence. *Journal of Plant Growth Regulation*, 33(1), pp.21–33.
Nakajima, M. et al., 2006. Identification and characterization of Arabidopsis gibberellin receptors. *Plant Journal*, 46(5), pp.880–889.
Nester, E.W., 2015. Agrobacterium: nature's genetic engineer. *Frontiers in Plant Science*, 5(January), pp.1–16. Available at: http://journal.frontiersin.org/article/10.3389/fpls.2014.00730/abstract.
Newman, R.H., Hill, S.J. & 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(4), pp.1558–1567. Available at: http://www.plantphysiol.org/cgi/doi/10.1104/pp.113.228262.
Nilsson, J. et al., 2008. Dissecting the molecular basis of the regulation of wood formation by auxin in hybrid aspen. *The Plant cell*, 20(4), pp.843–55. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2390731&tool=pmcentrez&rendertype=abstract [Accessed July 14, 2014].
Nishiyama, Y., 2009. Structure and properties of the cellulose microfibril. *Journal of Wood Science*, 55(4), pp.241–249.

Normanly, J., 2010. Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. *Cold Spring Harbor perspectives in biology*, 2(1), pp.1–17.

Nuruzzaman, M. et al., 2010. Genome-wide analysis of NAC transcription factor family in rice. *Gene*, 465(1–2), pp.30–44. Available at: http://dx.doi.org/10.1016/j.gene.2010.06.008.

Obudulu, O. et al., 2016. Quantitative proteomics reveals protein profiles underlying major transitions in aspen wood development. *BMC Genomics*, pp.1–17. Available at: http://dx.doi.org/10.1186/s12864-016-2458-z.

Ohman, D. et al., 2012. MYB103 is required for FERULATE-5-HYDROXYLASE expression and syringyl lignin biosynthesis in Arabidopsis stems. *The Plant journal : for cell and molecular biology*, 1, pp.63–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22967312 [Accessed June 16, 2014].

Oliver, L., 2018. China has sent 60,000 soldiers to plant trees. *World Economic Forum*. Available at: https://www.weforum.org/agenda/2018/02/china-army-soldiers-plant-trees/ [Accessed March 15, 2018].

Olsen, A.N. et al., 2005. NAC transcription factors: Structurally distinct, functionally diverse. *Trends in Plant Science*, 10(2), pp.79–87.

Otsuka, M. et al., 2004. Emission of ent-kaurene, a diterpenoid hydrocarbon precursor for gibberellins, into the headspace from plants. *Plant Cell Physiol*, 45(9), pp.1129–1138. Available at: http://pcp.oxfordjournals.org/cgi/content/full/45/9/1129.

Overvoorde, P. et al., 2005. Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana. *The Plant Cell*, 17(December), pp.3282–3300. Available at: http://www.plantcell.org/content/17/12/3282.short.

Parry, G. et al., 2009. Complex regulation of the TIR1/AFB family of auxin receptors. *Proceedings of the National Academy of Sciences*, 106(52), pp.22540–22545. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.0911967106.

Paz-Ares, J. et al., 1987. The regulatory c1 locus of *Zea mays* encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. *The EMBO journal*, 6(12), pp.3553–3558. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=553820&tool=pmcentrez&rendertype=abstract.

Petersen, P.D. et al., 2012. Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants. , pp.1–19.

Pickett-Heaps, J., 1968. Xylem wall deposition: radioautographic investigations using lignin precursors. *Protoplasma*, 65, pp.181–205.

Porco, S. et al., 2016. Dioxygenase-encoding *AtDAO1* gene controls IAA oxidation and homeostasis in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, 113(39), pp.11016–11021. Available at: http://www.pnas.org/lookup/doi/10.1073/pnas.1604375113.

Proebsting, W.M. et al., 1992. Gibberellin Concentration and Transport in Genetic Lines of Pea : Effects of Grafting. *Plant Physiol*, 100(3), pp.1354–1360. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16653128.

Ragauskas, A., Chemical composition of wood. *Chemical composition of wood*. Available at: http://ipst.gatech.edu/faculty/ragauskas_art/technical_reviews/Chemical Overview of Wood.pdf [Accessed March 15, 2018].

Ragni, L. et al., 2011. Mobile gibberellin directly stimulates Arabidopsis hypocotyl xylem expansion. *The Plant cell*, 23(April), pp.1322–1336.

Roy, S., 2016. Function of MYB domain transcription factors in abiotic stress and epigenetic control of stress response in plant genome. *Plant Signaling & Behavior*, 11(1), p.e1117723. Available at: http://www.tandfonline.com/doi/full/10.1080/15592324.2015.1117723.

Sablowski, R.W.M. & Meyerowitz, E.M., 1998. A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. *Cell*, 92(1), pp.93–103.

Santner, A., Calderon-Villalobos, L.I.A. & Estelle, M., 2009. Plant hormones are versatile chemical regulators of plant growth. *Nature Chemical Biology*, 5(5), pp.301–307.

Sasaki Itoh, H., A. et al., 2003. Accumulation of Phosphorylated Respressor for Gibberellin Signaling in an F- box Mutant. *Science.*, 299(2003), pp.1896–1899.

Schneider, R. et al., 2016. Cellulose and callose synthesis and organization in focus, what's new? *Current Opinion in Plant Biology*, 34, pp.9–16.

Shao, H., Wang, H. & Tang, X., 2015. NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. *Frontiers in Plant Science*, 6(October), pp.1–8. Available at: http://journal.frontiersin.org/Article/10.3389/fpls.2015.00902/abstract.

Silverstone, a L. et al., 1997. Developmental regulation of the gibberellin biosynthetic gene GA1 in Arabidopsis thaliana. *The Plant journal : for cell and molecular biology*, 12(1), pp.9–19. Available at: http://onlinelibrary.wiley.com/doi/10.1046/j.1365-313X.1997.12010009.x/abstract%5Cnhttp://onlinelibrary.wiley.com/store/10.1046/j.1365-313X.1997.12010009.x/asset/j.1365-313X.1997.12010009.x.pdf?v=1&t=hklcgkpy&s=acbd629e1ade0f0c4d62deede2dd751c5ac45fad.

Simon, S. & Petrášek, J., 2011. Why plants need more than one type of auxin. *Plant Science*, 180(3), pp.454–460.

Smalle, J. & Vierstra, R.D., 2004. the Ubiquitin 26S Proteasome Proteolytic Pathway. *Annual Review of Plant Biology*, 55(1), pp.555–590. Available at: http://www.annualreviews.org/doi/10.1146/annurev.arplant.55.031903.141801.

Sorce, C. et al., 2013. Hormonal signals involved in the regulation of cambial

activity, xylogenesis and vessel patterning in trees. *Plant Cell Reports*, 32(6), pp.885–898.
Sperry, J.S., 2003. Evolution of Water Transport and Xylem Structure. *International Journal of Plant Sciences*, 164(S3), pp.S115–S127. Available at: http://www.journals.uchicago.edu/doi/10.1086/368398.
Strabala, T.J., 2006. Gain-of-Function Phenotypes of Many CLAVATA3/ESR Genes, Including Four New Family Members, Correlate with Tandem Variations in the Conserved CLAVATA3/ESR Domain. *PLANT PHYSIOLOGY*, 140(4), pp.1331–1344. Available at: http://www.plantphysiol.org/cgi/doi/10.1104/pp.105.075515.
Sun, X. et al., 2010. N-terminal domains of DELLA proteins are intrinsically unstructured in the absence of interaction with GID1/gibberellic acid receptors. *Journal of Biological Chemistry*, 285(15), pp.11557–11571.
Sundberg, B., Uggla, C., and Tuominen, H., 2000. Cambial growth and auxin gradients. In and R. N. R. Savidge, J. Barnett, ed. *Cell and Molecular Biology of Wood Formation*. BIOS Scientific Publishers, pp. 169–188.
Takada, S. et al., 2001. The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. *Development (Cambridge, England)*, 128(7), pp.1127–35. Available at: http://dev.biologists.org/content/128/7/1127.abstract.
Thiman, K. V., 1935. On the plant growth hormone produced by Rhizopus suinus. *Journal of Biological Chemistry*, 109, pp.279–291.
Timell, T.E., 1967. Recent progress in the chemistry of wood hemi-celluloses. *Wood Sci. Technology*, 1, pp.45–70.
Ueguchi-Tanaka, M. et al., 2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature*, 437(7059), pp.693–698.
Ueguchi-Tanaka, M. et al., 2007. Molecular Interactions of a Soluble Gibberellin Receptor, GID1, with a Rice DELLA Protein, SLR1, and Gibberellin. *the Plant Cell Online*, 19(7), pp.2140–2155. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.106.043729.
Uggla, C. et al., 1996. Auxin as a positional signal in pattern formation in plants. *Proceedings of the National Academy of Sciences of the United States of America*, 93(17), pp.9282–9286.
Uggla, C., Mellerowicz, E.J. & Sundberg, B., 1998. Indole-3-Acetic Acid Controls Cambial Growth in Scots Pine by Positional Signaling1. *Plant physiology*, 117(1), pp.113–121.
Vidal, E.A. et al., 2010. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, 107(9), pp.4477–4482. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.0909571107.
Vollbrecht, E. et al., 1991. The developmental gene Knotted-1 is a member of a maize homeobox gene family. *Nature*, 350(6315), pp.241–243. Available at: http://www.nature.com/doifinder/10.1038/350241a0.
Wang, H. et al., 2010. Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous

plants. *Proceedings of the National Academy of Sciences*, 107(51), pp.22338–22343. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.1016436107.

Watanabe, Y. et al., 2015. Visualization of cellulose synthases in Arabidopsis secondary cell walls. *Science*, 350(6257), pp.198–203. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.aac7446.

Wilkerson, C.G. et al., 2014. Monolignol Ferulate Transferase Introduces Chemically Labile Linkages into the Lignin Backbone. *Science*, 344(6179), pp.90–93. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1250161 [Accessed April 4, 2014].

Willige, B.C. et al., 2007. The DELLA Domain of GA INSENSITIVE Mediates the Interaction with the GA INSENSITIVE DWARF1A Gibberellin Receptor of Arabidopsis. *the Plant Cell Online*, 19(4), pp.1209–1220. Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.107.051441.

Yabuta, T. and Sumiki, Y., 1938. On the crystal of gibberellin, a substance to promote plant growth. *J. Agric. Chem. Soc. Japan*, 14, p.1526.

Yamaguchi, S., 2008. Gibberellin metabolism and its regulation. *Annual review of plant biology*, 59(4), pp.225–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18173378.

Ye, Z.H. & Zhong, R., 2015. Molecular control of wood formation in trees. *Journal of Experimental Botany*, 66(14), pp.4119–4131.

Zhao, H. et al., 2017. The Arabidopsis thaliana Nuclear Factor Y Transcription Factors. *Frontiers in Plant Science*, 7(January), pp.1–11. Available at: http://journal.frontiersin.org/article/10.3389/fpls.2016.02045/full.

Zhao, Y., 2010. Auxin biosynthesis and its role in plant development. *Annu Rev Plant Biol*, 61, pp.49–64.

Zhong, R. et al., 2008. A Battery of Transcription Factors Involved in the Regulation of Secondary Cell Wall Biosynthesis in Arabidopsis. *The Plant Cell*, 20(10), pp.2763–2782. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2590737&tool=pmcentrez&rendertype=abstract [Accessed March 10, 2013].

Zhong, R. et al., 2011. Dissection of the transcriptional program regulating secondary wall biosynthesis during wood formation in poplar. *Plant physiology*, 157(3), pp.1452–68. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3252164&tool=pmcentrez&rendertype=abstract [Accessed June 13, 2013].

Zhong, R., Lee, C. & Ye, Z.-H., 2010. Global analysis of direct targets of secondary wall NAC master switches in Arabidopsis. *Molecular plant*, 3(6), pp.1087–1103. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20935069 [Accessed March 15, 2013].

Zhong, R. & Ye, Z.H., 2015. Secondary cell walls: Biosynthesis, patterned deposition and transcriptional regulation. *Plant and Cell Physiology*, 56(2), pp.195–214.

6 Popular science summary

Trees and their wood have played important roles in the history of our planet and species. Humans have used wood for a multitude of purposes from fuel to paper, biofuels, and lately; new bio-based materials.

For most people, a tree is just a tree, but the structure and composition wood greatly affects its characteristics. Since these decide how well suited a tree is for a certain industrial process, it means that characteristics that are good for tree fitness in the wild are not necessarily optimal for industrial use.

Starting in the 20th century, several large scale traditional breeding programmes for tree species were initiated, and have garnered some success, particularly in fast-growing, short rotation, species such as Eucalyptus. Research has shown that many of the interesting traits for industry are often complex and difficult to alter by traditional breeding methods.

New biotechnology methods, particularly genetic engineering, could be applied to make specific changes to the genomes of trees. However, to ensure that these changes affect development at the correct time and place requires in depth knowledge of the normal control of gene expression during wood formation.

In this thesis I present new knowledge about how the plant hormones auxin and gibberellin control the activity of factors directing the formation and development of fibre cells in wood.

7 Populärvetenskaplig sammanfattning

Träd och deras ved har spelat en viktig roll under både vår planets och arts historia. Vi människor har använt ved för en mängd ändamål, från bränsle för matlagning till papper, biobränslen och på senare tid: för framställning av nya biomaterial.

För de flesta är ett träd bara ett träd, men uppbyggnad av ved och dess sammasättning påverkar dess egenskaper i grunden. Eftersom dessa har stor inverkan på hur väl trädet lämpar sig för varje användningsområde innebär det att egenskaper som är fördelaktiga för träd i det vilda inte alltid är optimala för industriella processer.

Med början under 1900-talet startades flera storskaliga avelsprogram för skogsträd och dessa har nått stora framgångar, särskilt i snabbväxande arter som Eukalyptus. Forskning har dock visat att många av de egenskaper som industrin är intresserad av att påverka är komplexa och svåra att påverka med klassisk avel.

Nya gentekniska metoder skulle kunna tillåta specifika modifieringar av träds gen-uppsättning, men för att vara säker på att dessa får effekt på rätt tid och plats i växten krävs en god förståelse för när och var ved-specifika gener normalt är aktiva.

I den här avhandlingen lägger jag fram ny kunskap kring hur växthormonerna auxin och gibberellin reglerar aktiviteten hos faktorer som styr utvecklingen av fiberceller i ved.

8 Acknowledgements

I would like to start by acknowledging the contributions of my supervisors, **Dr. Urs Fischer** and **Professor Dr. Björn Sundberg**, for giving me the opportunity to take on this project. **Urs**, you have always been open for discussion and there to help me out, even when my ideas may have clashed with yours, you've heard me out and let me go my own way, which I appreciate.

Björn, even though our actual meetings have been rather few, I have immensely appreciated the opportunities to interact with you, and others from Stora Enso, and (attempt to) explain what I'm working on to others outside academia. It has greatly helped me develop my presentation and communication skills and allowed me to re-evaluate my own work from another perspective, which has been very valuable. A special thanks to you as well for your efforts with the NIR-project in Beihai.

I would like to acknowledge the support of my host company **Stora Enso**, in particular the work of **Johan Lindman**, and the **Berzelii Centre** for their financial support and enabling this project.

A special recognition is also due to Professor **Dr. Harry X. Wu** for organizing the **Industrial Research School of Forest Genetics, Biotechnology and Breeding**, as well as to my graduate student colleagues in the research school for making our various trips and courses so memorable.

Through my life I have had the immense good fortune of having teachers who have supported and encouraged me to be more and to do better. All of them are worthy of praise, however. I wish to name a few (in order of acquaintance): **Margareta Naurén**, who was the first to challenge me to go beyond the curriculum and explore the origin of life on earth in a "special

essay", encouraging me to take on the responsibility of finishing the work on my own time, with no schedule and only a final deadline (not unlike this thesis). **Thomas Carlsson**, my secondary school societies-teacher, who by his steady character and methodological approach greatly encouraged a scientific mind-set. To **Odd Minde** and **Christina Söderlund**, my high school math/chemistry, and biology, teacher respectively; I extend my sincerest gratitude, for their ground-breaking work establishing Rymgymnasiet (my high school), to their tireless efforts teaching and taking care of us children lost in the darkness of Kiruna. I would like to credit **Christina** especially, with instilling in me a great interest in biology, which directly led me to study biotechnology at Umeå University. I would like to recognize the great contribution to my education by **Dr. Veronica Albrecht-Borth**, who supervised me during my masters' thesis, through whom I learned many of the practical ins-and-outs of working in a research lab, and provided a model of how to conduct science. I would also like to thank **Dr. Jakob Prestele**, who taught me much about being a scientist, and planted the seed for my wanting to pursue a Ph.D. myself. And of all the colleagues at UPSC, Dr. **Judith Felten** is probably the one I've bugged most with questions throughout my PhD; thank you for all your help!

Of course, none of this would have been possible without the nurture and support of my amazing parents **Sune** and **Sirpa**, who have (without getting too self-congratulatory) made an absolutely fantastic job raising me. You not only let me go my own way at the early age of 16, but have always been there to help and support me, and even though I haven't always needed it; the knowledge that such support is always available has let me go through life largely un-troubled by whatever the world threw at me. And though I may never be able to explain the specifics of this book in a way that you'll find nearly as interesting as I would like; I hope you can appreciate it, and your contribution to making it happen.

Naturally, life as a Ph.D.-student would be tedious and dull without friends as great as mine. You truly are what have made living and working in Umeå worthwhile. Though many of you have moved away over the years, in many ways you have all become family to me and while I sadly can't list you all here, I'd like to give a shout-out to a few of you, in no particular order. So don't flatter yourself for being first **Henrik Vestin** - the one friend who can always be trusted to have my (in his view) best interest at heart, my very own devil-on-the-shoulder. Conversely, **Dr. Pär "Pumbaa" Jansson**, I truly am sorry for giving you that nickname, but it was you or I -and I had to act quickly

before it got pinned on me! Together, you and Henrik have helped guide me toward a moral centre, of sorts, in many things. Not forgetting ensuring good times a-aplenty, and for that, I thank you. **Erik Ortman**, as one of my oldest friends, we have managed to be classmates, corridor-neighbours, soldiers, and flatmates together since we met in high school, and with but one exception (involving a brand of alcohol, which shall remain nameless) I have only pleasant memories of you, thank you for all the great times, let's make it many more in the years to come!

Knut Bugge-Jakobsen and **Martin Johansson**, partners in crime, fellow food and whisky enthusiasts, thanks for your company and friendship, whether it's been brunches, "vinylkväll" or movie nights, there hasn't been a dull moment (except maybe the day after some Ubongo and over-violent air-drum solos).

Simon and **Lucy**, I want to thank you for being, not only, absolutely terrific people, but for all the delicious food. Simon especially should be praised for his great help in editing this thesis, ensuring my English is up to par.

Of course you've also played (pun intended) a great part in our Dungeons and Dragons game together with **Erik**, **Niklas**, **Markus** and **Marti**. More a food/beer club than your stereotypical collection of basement-nerds, it certainly has helped me keep my sanity (jury's still out though) over the past year and half.

And finally, to the members of the previously described (so-called) "**cool table**" (Kucukoglu, 2015): Thank you for all the fika, 3h lunches, "scientific" discussions and overall good times.

I know I have left many deserving people out of this section and I want you to know that you are not forgotten and I greatly appreciate you, and the joy you have brought to my life.