UMEÅ UNIVERSITY

DECODING LIGNIN IN SWEDISH ASPEN

Paths to Better Feedstocks and Resilient Trees

Mikko Luomaranta

Department of Plant Physiology
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To my wife, Suvi, and my children, Iisa and Iivo, for their love, support, and inspiration throughout this journey.

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Abstract

Trees are vital to our environment because they support biodiversity, carbon sequestration, oxygen production, and many other environmental functions. The lignocellulosic biomass produced by trees is also a renewable source of green products that can replace fossil fuel-derived products. More recently, their importance has been recognized as carbon sinks that assimilate atmospheric carbon dioxide into organic biomass. Climate change will expose trees to various environmental stresses and pathogens, and due to their sessile nature, trees rely on genetic diversity to survive and adapt. For instance, natural variation in resistance to pathogens allows trees to pass important resistance factors to their progeny and facilitate adaptation. Genome-wide methods have been developed to elucidate the molecular mechanisms underlying natural variation in important tree traits, which could be used in breeding for improved forest feedstocks.

In this thesis, the Swedish Aspen collection of *Populus tremula* trees (the SwAsp collection) was used as a resource to study natural variation in traits influencing tree biomass accumulation, pathogen resistance, and biomass processability. In addition, a systems genetic approach, including genome-wide analysis of expression quantitative trait loci (eQTL) and genome-wide association studies (GWAS), was taken to elucidate factors influencing variation in lignin biosynthesis in the SwAsp population. We identified biomass traits, in particular stem diameter and height, as the most critical factors influencing overall saccharification yield in this population based on multivariate analyses. We uncovered new regulatory aspects of lignin biosynthesis. Through GWAS, we detected genetic associations for saccharification, guaiacyl (G)- and syringyl (S)-type lignin subunits in young ramets and fungal resistance, providing potential

molecular markers for these traits. We also validated parts of our results using reverse genetics and an independent aspen collection. Finally, two soft rot fungal genera, *Ascocoryne* and *Cadophora*, were identified as highly abundant fungal pathogens in the ramets of the SwAsp trees. The symptoms of the fungal infections varied within the SwAsp population, and their extent correlated positively with the abundance of the *p*hydroxyphenyl (H)-type lignin.

This thesis highlights natural variation in traits significant for forest tree improvement, such as biomass accumulation, wood traits, and pathogen resistance, within the Swedish aspen population. It also provides details that help to understand lignin biosynthesis and fungal resistance in deciduous trees cultivated in short-rotation plantations. The identification of genetic and molecular markers for many of these traits contributes to efforts in tree breeding to enhance the resilience and utility of forest trees in the face of climate change.

Enkel sammanfattning på svenska

Träd är avgörande för vår miljö eftersom de stödjer biologisk mångfald, kolbindning, syreproduktion och många andra ekosystemtjänster. Den lignocellulosiska biomassan som produceras av träd är dessutom en förnybar resurs för biobaserade produkter som kan ersätta fossilbaserade produkter. På senare tid har deras roll som kolsänkor som assimilerar atmosfärisk koldioxid till organisk biomassa fått ökad uppmärksamhet. Klimatförändringarna kommer att utsätta träd för olika miljömässiga påfrestningar och patogener, och på grund av sin orörliga natur är träd beroende av genetisk variation för att överleva och anpassa sig. Exempelvis möjliggör naturlig variation i patogenresistens att träd kan

överföra viktiga resistensfaktorer till sina avkommor och därigenom underlätta anpassning. Genomomfattande metoder har utvecklats för att kartlägga de molekylära mekanismerna bakom naturlig variation i viktiga trädgenskaper, vilket kan användas i förädling för förbättrade skogsråvaror.

I denna avhandling användes den svenska asp populationen av *Populus tremula* (SwAsp-populationen) som en resurs för att studera naturlig variation i egenskaper som påverkar trädens biomassaproduktion, patogenresistens och enzymatisk sackarifikation av biomassan. Dessutom tillämpades ett systemgenetiskt angreppssätt, inklusive genomomfattande analyser av uttryckskvantitativa lokus (eQTL) och genomomfattande associationsstudier (GWAS), för att kartlägga faktorer som påverkar variation i ligninbiosyntesen i SwAsp-populationen. Vi identifierade biomassaegenskaper, mer specifikt stammens diameter och höjd, som de mest avgörande faktorerna för det totala sackarifikationsutbytet i denna population, baserat på både multivariata analyser och GWAS. Vi upptäckte nya regleringsmekanismer för ligninbiosyntesen, och genetiska associationer identifierades för sackarifikation, guaiacyl- (G) och syringyl- (S) ligninsubenheter i unga plantor samt svampresistens, vilket erbjuder potentiella molekylära markörer för dessa egenskaper. Vi validerade också delar av våra resultat med hjälp av transgenteknologi och en oberoende asp population. Slutligen identifierades två soft rot svampar, *Ascocoryne* och *Cadophora*, som de mest förekommande patogener i unga SwAsp träd. Symptomen av svampinfektionen varierade inom SwAsp-populationen, och deras omfattning korrelerade positivt med förekomsten av *p*-hydroxyfenyl- (H) lignin.

Denna avhandling lyfter fram naturlig variation i viktiga egenskaper för förbättring av skogsträd, såsom biomassatillväxt, vedegenskaper och patogenresistens, inom den svenska asp populationen. Den bidrar också till förståelsen av ligninbiosyntes och svampresistens i lövträd som odlas under korta omloppstider. Identifieringen av genetiska och molekylära markörer för många av dessa egenskaper stärker arbetet med trädförädling för att förbättra skogsträdens motståndskraft och användbarhet i ett föränderligt klimat.

Abbreviations

BLUP	Best linear unbiased predictor
C3'H	Coumaroyl 3′-hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
4CL	4-coumarate:CoA ligase
CCR	Cinnamoyl CoA reductase
CoA	Coenzyme A
CCoAOMT	Caffeoyl-CoA O-methyltransferase
COMT	Caffeic acid O-methyltransferase
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR-associated protein 9
CSE	Caffeoyl shikimate esterase
CWPO	Cell-wall-bound peroxidase
DNA	Deoxyribonucleic Acid
EMMA	Efficient Mixed Model Association
eQTL	Expression quantitative trait loci
F5H/Cald5H	Ferulate 5-hydroxylase/coniferaldehyde 5-hydroxylase
FDR	False discovery rate

FLA	Fasciclin-like arabinogalactan-protein
GEMMA	Genome-wide Efficient Mixed Model Analysis
GLM	General linear model
GS	Genomic Selection
G-type lignin	Guaiacyl-type lignin
GWAS	Genome-wide association study
HB	Homeobox protein
HCT	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase
H-type lignin	*p*-hydroxyphenyl type lignin
ITS	internal transcribed spacer
LAC	Laccase
LCC	Lignin carbohydrate complex
LLM	Linear mixed model
MFA	Microfibril angle
MYB	Myeloblastosis
NAC	NAM, ATAF1/2, CUC2
NST	NAC secondary wall thickening promoting factor
OPLS	Orthogonal projections to latent structures
PAL	Phenylalanine ammonia-lyase
PRX	Peroxidase
Py-GC/MS	Pyrolysis-gas chromatography/mass spectrometry
QTL	Quantitative trait loci
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RPI	Ribose-5-phosphate isomerase-like
SND	Secondary wall-associated NAC domain protein
SNP	Single nucleotide polymorphism
SSR	Simple sequence repeats
S-type lignin	Syringyl-type lignin

SwAsp	Swedish Aspen collection
TF	Transcription factor
TWG	Total wood glucose yield
VCF	Variant Call Format
VND	Vascular-related NAC domain protein
WND	Wood-associated NAC domain protein

List of publications and authorship contribution

Paper I. Escamez, S., Robinson, K. M., Luomaranta, M., Gandla, M. L., Mähler, N., Yassin, Z., Grahn, T., Scheepers, G., Stener, L.-G., Jansson, S., Jönsson, L. J., Street, N. R., & Tuominen, H. (2023). Genetic markers and tree properties predicting wood biorefining potential in aspen (*Populus tremula*) bioenergy feedstock. *Biotechnology for Biofuels and Bioproducts, 16*, Article 65. Available at: https://doi.org/10.1186/s13068-023-02363-4

I participated in the phenotypic characterizations and assisted in the analysis of the GWAS data and the manuscript writing.

Paper II. Luomaranta, M., Grones, C., Choudhary, S., Milhinhos, A., Kalman, T. A., Nilsson, O., Robinson, K. M., Street, N. R., & Tuominen, H. (2024). Systems genetic analysis of lignin biosynthesis in *Populus tremula*. *New Phytologist* 243(6):2157-2174. Available at: https://doi.org/10.1111/nph.19993

I participated in collection of the SwAsp samples in the field. I conducted the RNA-seq data analysis, carried out the GWAS, eQTL, and network analyses. I collected and analysed the UmAsp population. I wrote the manuscript with the help of HT and co-authors.

Paper III. Luomaranta, M., Schneider, A., Grones, C., Street, N. R., Robinson, K. M., & Tuominen, H. Abundant fungal infections in woody tissues of aspen ramets. (manuscript)

I participated in collection of the SwAsp samples in the field. I prepared samples for amplicon sequencing and chemical characterization of the wood. I did the analysis of the RNAseq data. I participated in the amplicon sequencing data analysis. I wrote the manuscript with the help of HT and the co-authors.

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1. Background

Wood has been invaluable throughout human history. It was, and still is, used as a building material to provide shelter and burned to provide heat energy. As civilizations advanced, wood was used as a durable and versatile material for ships and tools. After the start of the Industrial Revolution, coal replaced wood as an energy source, while brick and iron were the choices for building materials. However, due to increased awareness of the human impact on the environment and climate change, wood has recently experienced a renaissance as a sustainable material for construction (Lauri et al., 2017). Wood also stores carbon and provides a sustainable alternative to fossil-fuel-based products (Soimakallio et al., 2022). The question is whether the increased demand for wood can be met simultaneously when the forests increasingly suffer due to climate change (Ramsfield et al., 2016; Linnakoski et al., 2019) and wide-scale deforestation. It is estimated that one third of the forest land area has been lost since the Ice Age mainly due to human actions (Ritchie, 2021). The deforestation rate has gradually slowed since the peak years but is still lagging behind the goal of stopping deforestation by the year 2030 (Forest Declaration Assessment, 2022). More efficient use of woody biomass (Soimakallio et al., 2022), short-rotation tree practices, and higher product refinement (**Paper I**) could mitigate some of these issues.

Lignocellulosic raw materials can be refined into biofuels like methanol and ethanol. It requires large-scale harvests, transport from field to biorefineries, and chemical pretreatments (Hassan et al., 2019), resulting in much higher production costs than fossil fuels (Kargbo et al., 2021). Due to the renewable nature and more favorable carbon balance, organizations such as the European Union support biofuel production. EU is committed to a goal of 14% of the total transport fuel being derived from

advanced biofuels and biogas by 2030 (European Court of Auditors, 2023), which aligns with the goal of net-zero carbon emissions by 2050 (European Commission, 2020). However, to remain a viable source of energy production, the feedstocks need to be sustainable, and the efficiency of biomass conversion to biofuels needs improving.

Combining sustainability and efficiency is a challenge for the forest industry. This industry is critical for countries such as Finland, which has the largest proportion of forest land area in the EU (66%) and where the forestry and logging industry's gross value to GDP is tenfold the average of the EU (Eurostat, n.d). In 2022, Finland's gross value added (GVA) by the forestry and logging industry was the highest in the EU (1.64%), while Sweden's GVA was 0.35% (Eurostat, n.d).

Efforts to improve forest productivity by tree breeding started for conifers in the Nordic countries in the 1940s and 1950s, relying initially on the selection of plus trees (Haapanen et al., 2016). Currently, the conifer seedlings obtained from seed orchards in Scandinavia and Finland have 10-25% increased volume growth due to breeding (Ruotsalainen, 2014; Jansson et al., 2017). However, deciduous tree breeding has been less emphasized in the Nordic countries, and the focus has been primarily on native birch (Rosvall et al., 2001; Koski & Rousi, 2005). Although *Populus* species are fast-growing and potential biomass feedstock, there has not been much interest in the native aspen, while most of the efforts have focused on hybrid aspen (*P. tremula x P. tremuloides*) (Yu et al., 2001; Stener & Karlsson, 2004).

Tree breeding has certain limitations, of which the long breeding cycle may be the most important (Grattapaglia et al., 2018). It is particularly challenging in a changing climate, which can rapidly affect the growth

conditions of the trees and hence targets of tree breeding (Harfouche et al., 2014; Grattapaglia et al., 2018; Teshome et al., 2020). Due to climate change, trees are also predicted to be exposed to new pathogens and pests (La Porta et al., 2008; Ramsfield et al., 2016; Linnakoski et al., 2019). These challenges create a demand to accelerate the process of tree breeding.

Advances in next-generation sequencing allow cost-efficient wholegenome sequencing of new species and larger groups of individuals (Davey et al., 2011). These advancements have facilitated the emergence of genomic selection (GS) as a promising method to reduce breeding cycle time (Goddard & Hayes, 2007; Bhat et al., 2016; Grattapaglia et al., 2022). Genomic selection models have demonstrated reliable accuracy in predicting complex traits, including growth and wood quality, in tree species (Resende et al., 2012; Lenz et al., 2017). Even though genomic selection can be used to improve complex traits, it does not give information about the genetic architecture of the traits, raising the need for alternative methods, such as systems genetics.

Systems genetics seeks to understand how variations in DNA sequences contribute to complex traits by utilizing genome-wide association analysis (GWAS) and multiple 'omics' data (Civelek & Lusis, 2014). Lignin biosynthesis has been extensively studied in various *Populus* species using multiple layers of biological information, improving our understanding of this process (Wang et al., 2018; Xie et al., 2018; Balmant et al., 2020; Sulis et al., 2023). Lignin, a versatile component of plant cell walls, is a critical trait for biomass utilization as it has been recognized as a major recalcitrance factor in woody biomass (Ragauskas et al., 2014; Li et al., 2016).

1.1 *Populus* species as model organisms

The *Populus* genus comprises multiple species of poplars, cottonwoods and aspens (Tuskan et al., 2006). It has a broad geographical distribution from colder areas of the northern hemisphere to the Mediterranean (Liu et al., 2022). The first tree genome sequenced was *Populus trichocarpa* (Tuskan et al., 2006), which has been followed by the sequencing of multiple other *Populus* species including *Populus tremula* (Lin et al., 2018; Robinson et al., 2024), *Populus alba* (Ma et al., 2019), *Populus nigra* (Faivre-Rampant et al., 2016) and *Populus euphratica* (Ma et al., 2013). The available genomes and *Populus*-specific traits, such as fast growth, ease of vegetative propagation, and small genome size, have made the genus an established model organism within plant biology (Taylor, 2002; Sterky et al., 2004). *Populus* trees further provide a unique opportunity to dissect traits, such as perennial growth and seasonality, that are impossible to address in model organisms like *Arabidopsis thaliana* (commonly known as *Arabidopsis*) (Jansson & Douglas, 2007).

Studies of perennial traits testify to the success of using *Populus* species as a tree model system. Examples of this include the regulation of flowering time (Böhlenius et al., 2006; Hsu et al., 2006; Hsu et al., 2011; André et al., 2022) and seasonal traits such as bud burst (Hewett & Wareing, 1973; Heide, 2003; Pellis et al., 2004; Rinne et al., 2011; Yordanov et al., 2014; McKown et al., 2018) and leaf senescence (Andersson et al., 2004; Keskitalo et al., 2005; Lu et al., 2020; Fataftah et al., 2021). Many studies have also been done on wood properties that are particularly interesting traits for industries utilizing woody biomass (Hertzberg et al., 2001; Nilsson et al., 2008; Sundell et al., 2017; Abreu et al., 2020).

In Northern Europe, in countries like Sweden, European aspen (*P. tremula*) and other deciduous species have been historically considered less valuable than conifer species such as Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). The economic interest in conifers led to drastic policies in countries like Sweden, where deciduous trees were eradicated with extensive herbicide spraying carried out from 1948 to 1984 to suppress these species (Östlund et al., 2022), leading to the loss of many organisms hosted by the deciduous trees and a decline in forest biodiversity (Kivinen et al., 2020; Kusbach et al., 2024).

European aspen adapts well to diverse geo-climatic conditions and is widely distributed across much of Europe and Asia (Caudullo & de Rigo, 2016). Studies have identified, for example, a large-effect locus on chromosome 10 that is important for the adaptation to high-latitude regions (Rendón-Anaya et al., 2021) and to the timing of the bud set (Hall et al., 2007). European aspen displays substantial nucleotide diversity (π), a value representing genetic diversity in a species, ranging from 0.0061 to 0.0082 in three different aspen collections (Robinson et al., 2024), in comparison to the North American aspen species *P. tremuloides* (π = 0.0017) and poplar species such as *P. balsamifera* (π = 0.0027) and *P. trichocarpa* (π = 0.003) (Ingvarsson, 2005; Olson et al., 2010; Ismail et al., 2012; Fahrenkrog et al., 2017a). The high level of nucleotide diversity indicates that the European aspen is a prime study target for understanding natural variation in tree-related traits.

1.2 Natural variation

Natural variation refers to the phenotypic variation between individuals of a species determined by genetic variation. The most commonly studied type of genetic variation is currently single-nucleotide polymorphisms

(SNPs) as these are easy to identify using high throughput DNA sequencing technologies. A SNP is a nucleotide variant that differs among individuals in a population. To be utilised in GWAS studies a SNP must occur in a sufficient proportion of individuals, with 1% frequency being a commonly applied minimum allele frequency threshold (Liu, 2007).

Genetic polymorphisms can have various consequences. Nonsense mutations and insertions or deletions in a coding region can lead to a truncated protein, creating a loss-of-function mutation. Changes in the protein amino acid sequence due to missense mutations can also cause a loss of function when the mutation occurs in an active or binding site. At the same time, it can also impact folding, creating a change-of-function mutation (Alonso-Blanco et al., 2009). Besides alterations in protein function, a nucleotide polymorphism can impact gene expression when located in various locations such as promoter binding site, enhancer region, untranslated regions (UTRs), and introns (Shastry, 2009; Shaul et al., 2017; Srivastava et al., 2017). SNPs can also impact DNA methylation, thus causing changes in genetic regulation (Alonso-Blanco et al., 2009).

Variation is essential for the adaptation and survival of species, exemplified by efficient local adaptation in response to environmental factors in several *Populus* species (de Carvalho et al., 2010; Keller et al., 2011; Oubida et al., 2015; Ingvarsson & Bernhardsson, 2019). Lack of genetic diversity can be detrimental to a species, which has been shown in cultivars of high-yielding single clones or in a population of closely related individuals (van de Wouw et al., 2010). In contrast to domesticated crops, forest trees display high levels of genetic diversity, particularly in outcrossing species with large ranges and long lifespans (Hamrick et al., 1981; Müller-Starck, 1997).

Genetic diversity is crucial for adaptation and has become an important resource for studying the genetic basis of key traits in natural populations. A method to connect the phenotype of the trait to a genetic basis is quantitative trait locus mapping (QTL), which takes advantage of the linkage between the variation in a phenotype and molecular markers within a population of genetically related plants. Molecular markers can consist of simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP) and more recently single nucleotide polymorphism (SNP) (Miles & Wayne, 2008). QTL analysis typically utilizes an F2 population originating from a cross of two parents that are highly divergent for the trait of interest. Alternatively, a full-sib F1 progeny can be utilized in species such as forest trees with a long generation time (Doerge, 2002; Miles & Wayne, 2008). QTL has been implemented in many studies in *Populus* species to explore traits like cell wall chemistry (Muchero et al., 2015), growth traits (Du et al., 2016), and stress (Du et al., 2022).

Limitations in generating full-sib or F2 progeny, along with advances in whole-genome sequencing, have contributed to the rise of Genome-wide association studies (GWAS) to replace QTL as the standard method to study natural variation. GWAS utilizes the same principle, but in contrast to QTL analysis relies on genetic variation within a large population of unrelated individuals (Visscher et al., 2012). GWAS relies on SNP for genotyping to achieve a genome-wide coverage.

The associations in early GWAS studies utilized single-locus approaches, including general linear models (GLM) (Yoosefzadeh-Najafabadi et al., 2022). However, these models came with caveats and did not consider confounding factors such as population structure or other sources of variation that can lead to the detection of false positive associations. In

contrast, linear mixed models (LMMs) address these limitations by assuming non-independence among individuals.

In addition to population structure, GWAS can be misleading due to the small effect size of an SNP, which makes detecting associations difficult, or due to clustered loci where non-causal SNPs can cause 'synthetic peaks' (Clauw et al., 2024). Therefore, it is very important to validate GWAS results with independent methods such as functional studies in transgenic plants. Examples of such validations in forest trees are still very rare (Xie et al., 2018).

1.3 Growth and wood formation

Plant growth occurs through meristems, composed of pluripotent stem cells continuously dividing and differentiating into specialized cells. Primary growth represents directional growth from the apical meristems in co-operation with external stimuli such as light and gravitropism, resulting in stem and root elongation. Secondary growth, on the other hand, is derived from the lateral meristems, such as the vascular cambium, resulting in radial growth (Fischer et al., 2019). Perennial woody plant growth can also be categorized into vegetative and reproductive growth. Young perennial trees such as *Populus* undergo an extensive juvenile phase (7-10 years) before transition to reproductive growth (Hsu et al., 2006). During this juvenile phase, young perennial trees grow rapidly to secure resources including light and water in an environment where they may face competition from other plants (Brunner & Nilsson, 2004; Grattapaglia et al., 2009).

The secondary xylem, commonly termed wood, is a vital tissue that provides mechanical strength and facilitates water transport in vascular plants (Myburg et al., 2013). Wood formation involves five phases: cell

division, cell expansion, secondary cell wall formation, lignification, and programmed cell death (PCD) (Ye & Zhong, 2015; Sundell et al., 2017; Meents et al., 2018). Cell division occurs in the meristematic cells of the vascular cambium where periclinal division of the cambial cells gives rise to the secondary xylem cells. Cell division is followed by cell expansion (Meents et al., 2018). During this phase, the relaxation of the primary cell wall precedes water intake, resulting in a significant increase in the volume of the newly formed xylem cells. To counter this, active uptake of solutes maintains turgor pressure to ensure the integrity of the expanding cells while new cell wall components are synthesized and deposited.

Following expansion, the xylem cells deposit cellulose and hemicellulose to form the secondary cell wall, which is comprised of three layers: S1, S2, and S3 (Mellerowicz & Sundberg, 2008; Figure 1a & b). The S1 and S3 layers are relatively thin, with a large microfibril angle (MFA). In contrast, the S2 layer is the thickest with a low MFA, the cellulose microfibrils being aligned nearly parallel to the cell elongation axis (Barnett & Bonham, 2004; Mellerowicz & Sundberg, 2008). The orientation of these microfibrils significantly influences the mechanical strength of the wood fibers (Timell, 1967; Zhong & Ye, 2015). Cells with more longitudinally aligned microfibrils (low MFA) are better equipped to resist high-tension forces. For instance, tension wood forming on the upper sides of eucalypt branches has a considerably lower MFA than wood on the lower sides (Washusen et al., 2005).

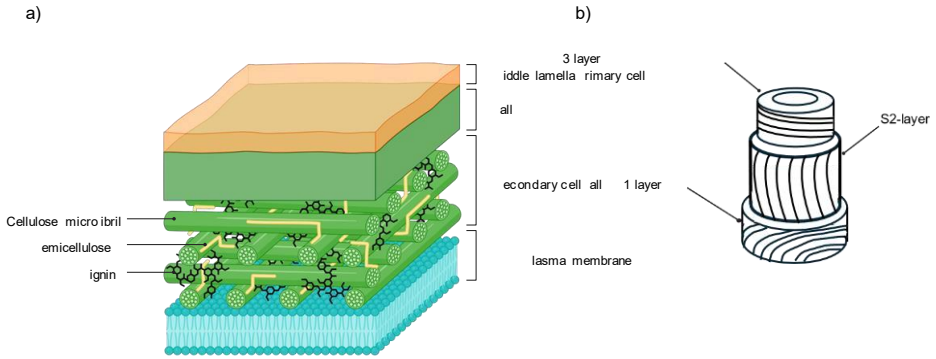

Figure 1. a) Schematic overview of the cell wall of a xylem element visualizing the key components; cellulose microfibrils (green), hemicellulose (yellow), and lignin (black). b) Visualization of the different layers of the secondary cell wall. The S2-layer shows low microfibril angle (MFA) in contrast to S1- and S3 layers. Figure 1a) was created in https://BioRender.com.

Lignification marks the next stage of wood formation, beginning during formation of the S1 layer of the secondary cell wall. During this process, oxidized lignin subunits are deposited into the cell wall, forming bonds with hemicellulose. Lignification is essential for physical support (Rathgeber et al., 2016) and provides cell walls with the hydrophobicity crucial for water transport.

In the final stage the xylem cells of the angiosperms, except for the ray parenchyma cells, undergo programmed cell death. This step is critical for converting these cells into fully functional xylem elements. Programmed cell death is, therefore, integral to xylem differentiation, enabling the development of mature xylem cells.

1.3.1 Wood polysaccharides

Since wood primarily comprises secondary cell walls, the chemical composition of the secondary cell walls largely determines its overall composition. Cellulose is the most abundant component in gymnosperms and angiosperms, making up 40-50% of the woody biomass (Timell, 1967). Cellulose is a linear polymer consisting of glucose subunits connected ith β-1,4-glycosidic linkages. Each glucose unit forms two hydrogen bonds within the linear polymer with its neighbors whereby the C3 and C6 hydroxyl groups donate hydrogen bonds to the next glucose unit's ring oxygen and C2 hydroxyl (McNamara et al., 2015). Cellulose is produced by cellulose synthase complexes at the plasma membrane, where glucan chains are secreted and aggregate into crystalline microfibrils. These microfibrils consist of 18 or 24 glucan chains and are important, for example, in determining the directionality of cell growth (Cosgrove, 2014).

Heteropolysaccharides hemicelluloses are the second most abundant polymer group in angiosperm trees. Hemicellulose chains consist mainly of glucose, mannose, and xylose monomers, while lateral chains often comprise monosaccharides such as arabinose and galactose (Scheller & Ulvskov, 2010). These polymers are synthesized in the Golgi apparatus by glycosyltransferases that catalyze the formation of glycosidic bonds (Schultz & Coleman, 2021). In the secondary cell walls of angiosperms, xylan is the most abundant hemicellulose, making up roughly 15 to 30% of the biomass, while glucomannans are less abundant at 3-4% (Timell, 1967). Hemicelluloses form non-covalent bonds with cellulose microfibrils, significantly influencing plant cell walls' structural integrity and mechanical properties (Simmons et al., 2016; Berglund et al., 2020; Jarvis, 2023).
They also form covalent bonds with the third major cell wall polymer, lignin (Nishimura et al., 2018), providing extra rigidity to cell walls.

1.4 Lignin

Lignin is a polyphenolic polymer that evolved roughly 450 million years ago with the evolution of vascular plants (Renault et al., 2019). The metabolic scaffold contains eight key monolignol biosynthesis genes that were established in the earliest land plants before the rise of the vascular plants; this core gene scaffold can be found in the moss *Physcomitrella patens*, tracing the origin of the genes responsible for monolignol biosynthesis to bryophytes. However, mosses do not have lignin, and the lignification as a process evolved only with the emergence of the tracheophytes (Weng & Chapple, 2010).

Lignin in the wood of vascular plants mainly consists of monolignol subunits: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). These subunits differ by the degree of methoxylation of the aromatic ring. They result from the polymerization of monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols (Boerjan et al., 2003; Vanholme et al., 2019; Chantreau & Tuominen, 2022).

The amounts and polymer composition vary widely between different plant families. Generally, monocot species display a higher abundance of H-type lignin in their lignocellulosic biomass (Lourenço & Perreira, 2018) than perennial woody plants. In gymnosperm species, lignin consists mostly of G-type subunits and a minor fraction of H-type lignin (Boerjan et al., 2003), which differs markedly from angiosperms, which have rather equal levels of S- and G-type lignin with the ratio between the two subunits varying between different species. In aspen the S to G ratio varied between 2 - 2.25 (**Paper I**; **Paper II**), while it was 3 for *Betula pendula* (Fagerstedt et al., 2015) and 3.8 in mature *Eucalyptus grandis* wood (Rencoret et al., 2011). In the wood of angiosperm species such as *Populus*, lignin is present in the cell walls of the fibers, which provide

structural support to the tree, in the vessels, where it imparts hydrophobicity for water transport, and in the ray parenchyma cells, which play an important role in various metabolic functions (Chantreau & Tuominen, 2022). The lignin composition also varies between different xylem cell types; the fibers being enriched in the S-type and the vessel elements in the G-type lignin (Gorzsás et al., 2011; Lourenço et al., 2016; Zhang et al., 2020). Furthermore, this composition changes during the maturation of the xylem elements (Ménard et al., 2022).

Perturbation of lignin content impairs water conductivity. Transgenic hybrid poplar trees with reduced lignin content had impaired water transport capacity and increased susceptibility to embolism (Kitin et al., 2010; Voelker et al., 2011a). Lignin composition was also shown to influence the hydraulic properties of the water-transporting elements as well as response to dehydration in *Arabidopsis* tracheary elements (Ménard et al., 2022).

The role of lignin as a structural support has become clear in mutants for the different lignin-biosynthetic genes, where the low lignin content has typically led to dwarfism (Anderson et al., 2015; Panda et al., 2020; Ha et al., 2021). For instance, a mutation in Cinnamoyl CoA reductase (CCR) causes a drastic reduction in lignin content and collapse of the xylem in *Arabidopsis* (Jones et al., 2001). Down-regulation o the coumaroyl 3′hydroxylase (C3'H) in hybrid poplar (*P. alba* × *P. grandidentata*) drastically reduced the lignin content and led to collapsed xylem (Coleman et al., 2008). In *P. tomentosa*, CRISPR-Cas9-mediated knock-out of C3'H3 resulted in significantly decreased G- and S-type lignin content, reduced stem height, and collapsed xylem (Zhang et al., 2023a), while hybrid poplar lines with the most suppressed expression of 4-coumarate:CoA ligases (4CL) exhibited irregular xylem vessels, reduced

wood stiffness and strength (Voelker et al., 2011b). These studies highlight the vital role lignin has on water transport and structural strength during normal development and growth of vascular plants.

1.4.1 Lignin biosynthesis

The lignin biosynthetic pathway has been studied extensively in both *Arabidopsis* (Vanholme et al., 2012a) and *Populus* (Shi et al., 2010; Wang et al., 2018; Sulis et al., 2023). Plants produce lignin via the phenylpropanoid pathway, whereby the first reaction is the enzymatic conversion of phenylalanine to cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL) (Figure 2). *P. trichocarpa* has five *PAL* genes, of which *PtrPAL2, 4,* and *5* are xylem specific (Shi et al., 2010). The importance of PAL was highlighted by a drastic decrease in the total lignin content of *P. trichocarpa* RNAi-lines downregulated for *PAL2, 4* and 5 (Wang et al., 2018). The *PtrPAL2* and *PtrPAL5* RNAi lines had the most reduced lignin content in this study. However, the *PtrPAL5* RNAi lines grew better than the *PtrPAL2* lines, which is in line with the more recent results from the CRISPR-Cas9 knockout lines for the *PAL* genes which revealed the most drastic changes in lignin content for the *PtrPAL2* knockout lines (Sulis et al., 2023). These results suggest that *PtrPAL5* could be used as a single gene target to decrease lignin content without significant growth tradeoffs (Wang et al., 2018).

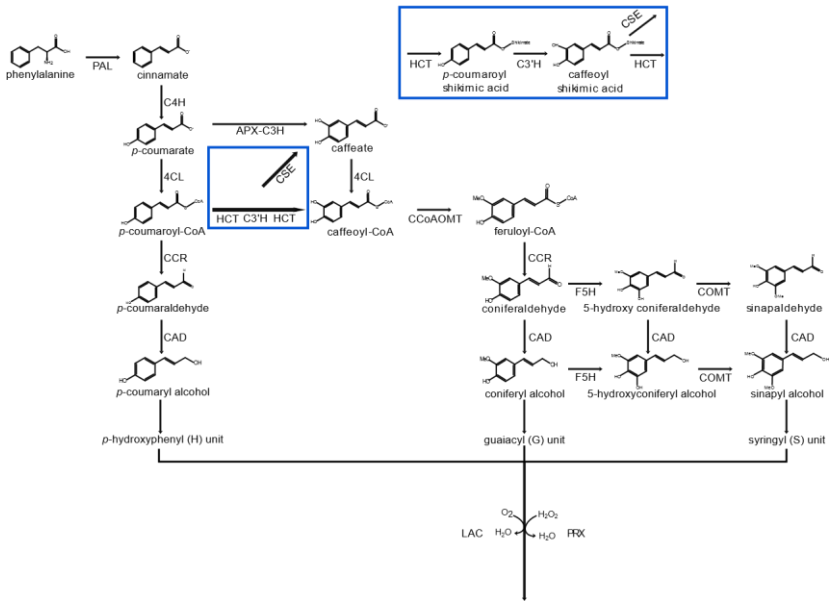

Figure 2. Key enzymes and metabolites in the lignin-biosynthetic pathway. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3’ , coumarate 3’-hydroxylase; CSE, caffeoyl shikimate esterase; CCR, cinnamoyl CoA reductase; COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F5H/Cald5H, ferulate 5hydroxylase/coniferaldehyde 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; LAC, laccase; PRX, peroxidase. Modified from Renström et al. (2024).

The next reaction in the pathway involves cinnamate 4-hydroxylase (C4H), which catalyzes the conversion of cinnamic acid to *p*-coumaric acid (4-hydroxycinnamic acid) by hydroxylating the 4-position of the aromatic ring (Figure 2). Besides monolignols, C4H is an essential enzyme for other

pathway derivatives, such as flavonoids and phytoalexins (Ehlting et al., 2006; Vanholme et al., 2019). There are three *C4H* genes in the *P. trichocarpa* genome (Kim et al., 2020a), and two of them (*PtrC4H1* & *PtrC4H2*) are involved in lignin biosynthesis of woody tissues (Wang et al., 2018). Simultaneous downregulation of *PtrC4H1* & *PtrC4H2* did not diminish the growth or change the lignin content compared to the wild type (Wang et al., 2018; Kim et al., 2020a). More detailed information was obtained from CRISPR-Cas9 lines, where knockout of *PtrC4H1* resulted in decreased G-type lignin content without a big impact on total lignin while the knockout of *PtrC4H2* led to a marked reduction in total lignin content and severe growth impairment compared to the wild type. The *PtrC4H2* knockout line also exhibited a significant decrease in the wood modulus of elasticity, highlighting the importance of C4H2 for normal development (Sulis et al., 2023).

The 4-coumarate:CoA ligases (4CL) convert several cinnamic acid derivatives to CoA thioesters (Figure 2). *P. trichocarpa* has 17 *4CL* genes (Shi et al., 2010), out of which *Ptr4CL3* and *Ptr4CL5* are considered to be involved in the lignin-biosynthesis of woody tissues based on xylemspecific expression and functional studies (Wang et al., 2018; Sulis et al., 2023; **Paper II**). Ptr4CL3 is considered to have the highest 4CL activity during lignification of woody tissues, and it has well-described orthologs in different species (Raes et al., 2003; Voelker et al., 2010; Vanholme et al., 2012a; Chen et al., 2014). Both Ptr4CL3 and Ptr4CL5 use 4-coumaric, caffeic, and ferulic acids as precursors, and Ptr4CL5 can also convert sinapic acid (Chen et al., 2014). Downregulation of the *Ptr4CL3* led to severe growth reduction in some of the *P. trichocarpa* RNAi lines even though lignin content was not severely affected (Wang et al., 2018). CRISPR-Cas9 mediated knockout lines in the single *Ptr4CL3* or *Ptr4CL5* in *P. trichocarpa* had roughly a 10% decrease in lignin content

compared to the wild type, and significantly increased H-type lignin content (Sulis et al., 2023).

HydroxyIcinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) catalyzes the deacylation of *p*-coumaroyl-CoA to *p*-coumaroyl shikimic acid as well as the conversion of caffeoyl shikimic acid to caffeoyl-CoA (Figure 2). Two out of ten *P. trichocarpa* HCTs, PtrHCT1, and PtrHCT6, are recognized for their role in the lignification of woody tissues (Shi et al., 2010; Wang et al., 2018; Sundell et al., 2017; **Paper II)**. CRISPR-Cas9 mediated knock-out of the single *PtrHCT1* or *PtrHCT6* resulted in significant increases in H-type lignin content, and even though the total content of the lignin did not differ significantly from the wild type, the growth of the trees was compromised (Sulis et al., 2023). A natural recessive allele in *P. nigra HCT1* introduces an early stop codon, leading to a truncated protein (Vanholme et al., 2013a), which in homozygous trees led to a substantial (17-fold) increase in H-type lignin content compared to ten different *P. nigra* genotypes without the *HCT1* mutant allele (Vanholme et al., 2013a). Reduced *HCT* expression in tobacco, alfalfa, and *Pinus radiata* reduced lignin content but increased H-type lignin content compared to control (Hoffmann et al., 2004; Shadle et al., 2007; Wagner et al., 2007), accentuating the role of HCTs in the regulation of lignin subunit composition.

Coumarate 3'-hydro ylase (C3') catalyzes the conversion o *p*-coumaroyl shikimic acid to caffeoyl shikimic acid by 3-hydroxylation of the aromatic ring and is speculated to form a complex with C4H (Chen et al., 2011; Gou et al., 2018; Kim et al., 2020a) (Figure 2). This gene family is reported to have at least four members in the *P. trichocarpa* genome (Shi et al., 2010), with *PtrC3´H3* being specifically expressed in the xylem tissues. Downregulation of *C3'H* decreased the G-type and drastically

increased the H-type lignin content in hybrid poplar with little impact on the S-type lignin (Peng et al., 2021). Knockout of the *PtrC3´H3* led to significant decreases in tree height, wood modulus of elasticity and lignin content compared to the wild type (Sulis et al., 2023). Interference with C3' function has also, in multiple species, led to a substantial increase in the H-type lignin content of the mutants (Ralph et al., 2006; Wang et al., 2018; Sulis et al., 2023), highlighting its role as an important enzyme in determining the lignin subunit composition.

The caffeoyl shikimate esterases (CSE) family was recently recognized in lignin biosynthesis (Vanholme et al., 2013b). CSEs catalyze the conversion of caffeoyl shikimate into caffeate in *Arabidopsis* and, together with 4CL, circumvent the second HCT reaction in the lignin pathway (Figure 2). *Populus* has two CSE genes, *CSE1* and *CSE2*, which have been studied in hybrid aspen (*P. tremula* x *P. alba*) (de Vries et al., 2021). Single knockouts of *CSE1* or *CSE2* did not reduce lignin content or influence tree growth, while double knock-out lines for both *CSE1* and *CSE2* showed reduced growth, changes in the xylem morphology, and reduction in the lignin content compared to the wild type (de Vries et al., 2021). Somewhat contrasting results were obtained in hybrid poplar (*P.alba* × *P. glandulosa*), where single knock-out *CSE* lines were shown to have significantly reduced lignin content (Jang et al., 2021). It is, therefore, possible that the two CSE genes are not completely redundant.

Cinnamoyl CoA reductase (CCR) catalyzes the reduction of cinnamoylCoA esters to cinnamaldehydes (Figure 2). The *Populus* genome contains nine (Shi et al., 2010) or ten *CCR* genes (Barakat et al., 2011); however, only PtrCCR2 has been directly associated with lignin biosynthesis of woody tissues (Shi et al., 2010). Downregulation or knocking out *PtrCCR2* reduced lignin content and led to severe growth

defects in *P. trichocharpa* (Wang et al., 2018) and hybrid aspen (*P. tremula* x *P. alba*) (De Meester et al., 2020). In addition, down-regulation of *CCR* in *P. tremula* × *P. alba* accumulated monolignol precursors into lignin structure and gave a distinct color to the xylem due to the accumulation of ferulic acids (Leple et al., 2007).

Caffeic acid O-methyltransferase (COMT) belongs to a versatile group of enzymes that methylates monolignol precursors at the 5- and 3-hydroxyl positions (Li et al., 2000) (Figure 2). Perturbation of *COMT* altered the syringyl to guaiacyl ratio in various species (Doorsselaere et al., 1995; Marita et al., 2001; Vanholme et al., 2010; Wang et al., 2018; Yoo et al., 2018), emphasizing the role of this enzyme in controlling the flux between G- and S-type lignin. Out of the 25 *P. trichocarpa COMT* genes (Shi et al., 2010), only *PtrCOMT2* has been assigned a role in lignin biosynthesis during normal wood formation (Shi et al., 2010**)**.

Caffeoyl-CoA O-methyltransferase (CCoAOMT) catalyzes the methylation of caffeoyl-CoA to feruloyl-CoA (Figure 2). The *Populus* genome has recently been reported to contain eight CCoAOMTs based on the Hidden-Markov Model (Zhao et al., 2022a), from which CCoAOMT1, CCoAOMT2, and CCoAOMT3 are linked to lignin
biosynthesis of woody tissues (Shi et al., 2010). In *P. trichocarpa*, downregulation of *PtrCCoAOMT* genes decreased lignin content and increased wood density (Wang et al., 2018). Knocking out the different *CCoAOMT* genes by CRISPR-Cas9 led to different phenotypes in *P. trichocarpa*. In comparison to the wild type, the knockout line of *PtrCCoAOMT1* had the lowest lignin content and the most severe growth reduction, while the knockout line of *PtrCCoAOMT2* showed the most significant changes in the subunit composition. The *PtrCCoAOMT3*

knockout line showed no drastic changes in growth or lignin content but decreased H-type lignin content (Sulis et al., 2023).

Ferulate 5-hydroxylase/coniferaldehyde 5-hydroxylase (F5H/Cald5H) catalyzes 5-hydroxylation of S-type lignin precursors (Humphreys et al., 1999) (Figure 2). The overexpression of *F5H* has been shown to lead to a substantial accumulation of S-type lignin in hybrid poplar (*P. tremula* x *P. alba*) (Stewart et al., 2009) and *P. tomentosa* (Jiang et al., 2021). *P. trichocarpa* has three *F5H/Cald5H* genes of which PtrF5H1 and PtrF5H2 have been reported most important in lignin biosynthesis in woody tissues on the basis of gene expression and functional studies (Shi et al., 2010; Wang et al., 2018; **Paper II)**. The impact of F5H on S-type lignin biosynthesis was evident in *P. trichocarpa* CRISPR-Cas9 knockout lines (Sulis et al., 2023). While the knockout of *PtrF5H1* significantly reduced both the S-type lignin content (down to 62.2% of the wild type) and the S/G ratio (52.4% of the wild type), the *PtrF5H2* knockout had an even more drastic impact with S-type lignin content dropping to 34% and the syringyl to guaiacyl ratio to 12% of the wild-type level. Both knockout lines exhibited reduced growth. However, saccharification of untreated biomass was greatly improved, supporting the notion that the syringyl to guaiacyl ratio influences biomass recalcitrance (Sulis et al., 2023).

Cinnamyl alcohol dehydrogenases (CAD) catalyze the final reaction in the lignin monomer biosynthesis. CADs reduce the hydroxycinnamyl aldehydes to corresponding monolignols. A total of 15 CAD genes have been identified in *P. trichocarpa* (Barakat et al., 2009). CADs are involved in several different biosynthetic processes, and in *P. trichocarpa PtrCAD1* and *PtrCAD2* have been indicated as the main CADs involved in lignin biosynthesis in wood (Shi et al., 2010; Wang et al., 2018). The *PtrCAD1* homolog in aspen has a high expression across different woody tissues,

while the *PtrCAD2* homolog has a distinct expression profile compared to the rest of the lignin-biosynthetic genes (Sundell et al., 2017; **Paper II)**. Interestingly, PtrCAD2 was reported to have specificity towards sinapyl aldehyde (Li et al., 2001) and it was even termed as sinapyl alcohol dehydrogenase (SAD), which was later challenged by enzyme kinetics studies indicating that although PtrCAD2 prefers sinapyl aldehyde over conifer aldehyde, it can reduce both substrates (Chao et al., 2014; Wang et al., 2014a). Down-regulation of *PtrCAD1* did not result in severe growth defects, whereas significant growth reductions were observed in lines where both *PtrCAD1* and *PtrCAD2* were down-regulated (Wang et al., 2018). In a separate study, down-regulation of *PtrCAD2* did not impact lignin content or growth but induced changes in the xylem cell morphology (Miller et al., 2019). CRISPR-Cas9 knockouts of the single *PtrCAD1* or *PtrCAD2* displayed wild type level growth, lignin content, and composition while the *PtrCAD1* & *PtrCAD2* double knockout had only a slightly reduced lignin content, increased syringyl to guaiacyl ratio and decreased H-type lignin without drastic effects on the growth of the trees (Sulis et al., 2023), suggesting that additional CADs might operate during vascular development.

1.4.2 Transcriptional regulation of the lignin-biosynthetic genes

The transcriptional regulation of lignin-biosynthetic genes has been well studied in different species, and has led to the identification of numerous transcription factors (TFs), particularly from the NAC (Zhong et al., 2007; Zhong et al., 2008; Zhong et al., 2010) and MYB families (Zhong et al., 2008; Tian et al., 2013; Li et al., 2015; Xie et al., 2018; Ohtani & Demura, 2019; Geng et al., 2020; Balmant et al., 2020; Kim et al., 2020b; Xiao et al., 2021). Several of these TFs are involved in the regulation of not only

lignification but the whole process of secondary cell wall formation. The secondary wall-associated NAC domain protein 1 (SND1; Zhong et al., 2006) and the Vascular-related NAC domain 6 and 7 (VND6 and 7; Kubo et al., 2005; Yamaguchi et al., 2011) were among the first TFs recognized as master regulators of secondary cell wall synthesis in *Arabidopsis*. In *Arabidopsis*, NAC transcription factors in class IIB contain VND (vasculararelated NAC domain protein), NST (NAC secondary wall thickening promoting factor)/SND (secondary wall-associated NAC domain protein) and SOMBRERO-related gene families. The homologous NAC family genes in *P. trichocarpa* (16 genes) have been named as VNS (VND-, NST/SND-, SMB-related proteins; Ohtani et al., 2011) or WND (woodassociated NAC domain transcription factors; Zhong et al., 2010). As master regulators of secondary cell wall formation, the *Populus* VNS/WND genes form a regulatory tier that controls the expression of downstream transcription factors (Ohtani et al., 2011).

Several MYB genes have been shown to regulate the expression of the lignin-biosynthetic genes. In *Arabidopsis*, MYB42 and MYB85 are important regulators of lignin biosynthesis during secondary cell wall formation (Zhong et al., 2008; Geng et al., 2020). In *P. tomentosa*, overexpression of *PtoMYB92*, a homolog of the *Arabidopsis MYB42* and *MYB85*, induced the expression of multiple lignin-biosynthetic genes and led to ectopic lignification (Li et al., 2015). In **Paper II**, we identified a central role in our lignin co-expression network for the homologous genes of MYB42 and MYB85. Similar findings were reported by Balmant et al. (2020), who identified MYB125, a homolog of MYB42 and MYB85, as a key regulator of lignin-biosynthetic genes in *P. deltoides*. In this report, eQTL analysis identified MYB125 as a trans-acting regulator of various lignin-biosynthetic genes, a finding further supported by the significant upregulation of these genes and increased lignin content in transgenic

lines overexpressing MYB125 (Balmant et al., 2020). **Paper II** and previous studies support the importance of the MYB42 and MYB85 and their homologous genes specifically in lignification. Various other MYBs have also been shown to influence lignin biosynthesis, including PtoMYB55 (Sun et al., 2020), PtoMYB74 (Li et al., 2018), PtoMYB92 (Li et al., 2015), PtoMYB156 (Yang et al., 2017), PtrMYB152 (Wang et al., 2014b), PtoMYB170 (Xu et al., 2017) and PtoMYB216 (Tian et al., 2013). In addition to VNS/WND and MYB transcription factors, new regulators are constantly recognized in lignin biosynthesis. The 5-enolpyruvylshikimate 3-phosphate synthase (PtrEPSP) suppresses a SLEEPER-like transcriptional regulator, which in turn is a repressor of the *Populus* homolog (PtrMYB021) of the *Arabidopsis* MYB46, a master regulator of the secondary cell wall formation (Xie et al., 2018). Overexpressing *PtrEPSP* in *P. deltoides* led to changes in the phenylpropanoid metabolites and altered lignin deposition.

1.4.3 Lignin polymerization

Lignin polymerization occurs through oxidative coupling of the monolignols (Tobimatsu & Schuetz, 2019). Two distinct gene families catalyze this reaction; laccases and peroxidases (Ralph et al., 2007; Tobimatsu & Schuetz, 2019; Figure 2). Laccases (LAC) belong to the group of multicopper oxidoreductases that catalyze monolignol oxidation, employing molecular oxygen as the final electron acceptor (Witayakran & Ragauskas, 2009; Kudanga et al., 2011). The *P. trichocarpa* genome has been reported to contain 53 laccase genes (Bryan et al., 2016; Xu et al., 2022). Functional studies on these laccases have demonstrated varying affinities for specific oxidation targets. For instance, PtrLAC23 exhibited a strong affinity for coniferyl alcohol (Liao et al., 2023), whereas PtrLAC16 preferred sinapyl alcohol (Liu et al., 2021). In *P. tomentosa*, PtoLAC14 showed a preference for coniferyl alcohol (Qin et al., 2020) and knocking

out the gene led to an increased S- to G-type lignin ratio in comparison to control, opposite to what was observed in the overexpression lines. These studies give important insight into the function of these laccases, showing that despite the large number of LAC genes they are not completely redundant but might serve rather specific functions.

Plant-specific Class III peroxidases (PRX) involved in lignification utilize hydrogen peroxide as an electron donor to oxidize monolignols (Passardi et al., 2004; Passardi et al., 2005; Battistuzzi et al., 2010; VedaP et al., 2017). *P. trichocarpa* is reported to have at least 93 PRX genes, expressed in various tissues (Ren et al., 2014). Besides lignification, peroxidases are involved in many processes such as plant defense (Hiraga et al., 2001; Almagro et al., 2009), seed germination, and stress resistance (Shigeto & Tsutsumi, 2016). A few peroxidases have been functionally characterised in lignification in *Populus*. The *P. alba* cell-wallbound peroxidase (CWPO-C) was capable of oxidizing large lignin oligomers (Aoyama et al., 2002; Sasaki et al., 2004). PtomtAPX, a class III mitochondrial ascorbate peroxidase, catalyzed lignin polymerization *in vitro* and stimulated lignin accumulation in transgenic of *P. tomentosa* trees (Zhang et al., 2022). This suggests that, despite the large number of peroxidases, they are not entirely redundant, and specific peroxidases have important roles in lignification.

1.4.4 Lignin recalcitrance

Saccharification of woody biomass entails the process of enzymatic hydrolysis of the polysaccharides of the woody biomass into fermentable sugars that can be utilized for biofuel, biochemical, and bioplastic synthesis. In particular, the fast growth of *Populus* species has made them a viable candidate to serve as a feedstock (Mola-Yudego et al., 2017) for

green fuel and products (Ragauskas et al., 2006; Mohr & Raman, 2013; Porth & Kassaby, 2015; Böhlenius et al., 2023).

Saccharification efficiency is limited by various physical and chemical "recalcitrance" actors (cCann & Carpita, 2015). ignin is one o the better-known recalcitrants, and high lignin content often correlates with increased recalcitrance, resulting in poor saccharification efficiency of the biomass (Li et al., 2016; Zoghlami et al., 2019). Lignin forms a physical barrier in the cell wall, which prevents the access of the hydrolytic enzymes to their cellulosic substrates in the cell walls (Vanholme et al., 2010; Vermaas et al., 2015). Lignin can also irreversibly adsorb the hydrolytic enzymes and therefore reduce the process efficiency (Kumar & Wyman, 2009; Zeng et al., 2014). In addition to lignin, high abundance of hemicelluloses and the lignin-carbohydrate (LCC) bonds between hemicelluloses and lignin contribute to the recalcitrance of the lignocellulosic biomass (Terrett & Dupree, 2019). To overcome these hurdles, pretreatments are normally applied to remove either the lignin or the hemicellulose (Zhu & Pan, 2010). In **Paper I**, we introduced a mild acidic treatment that efficiently converts hemicellulose into pentose sugars, potentially reducing cross-links between cell wall polymers and enhancing accessibility for enzymatic hydrolysis (Gandla et al., 2015). This technique also needs less energy as it does not require extreme temperatures. Even though pretreatments significantly improve biomass saccharification, these processes are costly and environmentally harsh.

Several efforts have been taken to improve saccharification efficiency by reducing lignin content by transgenic technology in various tree species. However, drastic modifications of lignin content often lead to compromised biomass yield, reducing the number of potential target genes (Chanoca et al., 2019; De Meester et al., 2022). Instead of modifying lignin amount,

the focus has more recently turned into modification of lignin content, as plants with higher S-type lignin content have been generally reported to have improved saccharification efficiency (Weng et al., 2010; Escamez et al., 2017; Yoo et al., 2018; Fan et al., 2020) even though contradictory results have also been reported (Meng et al., 2017). In the SwAsp collection of aspen trees, we detected a negative relationship between G-type lignin content and saccharification glucose yield (**Paper I**), which is in line with S-type lignin having a positive effect on saccharification efficiency in plants. Efforts into producing feedstocks with increased S-content with genetic breeding or transgenic technology are therefore tempting.

1.4.5 The role of lignin in abiotic stress

In addition to its essential role in growth and development, lignification is also affected by changes in response to various stress factors. Plants cannot move and thus are often exposed to environmental stresses. Abiotic stress includes drought, extreme temperatures, light, salinity, and a lack of nutrients (Moura et al., 2010; Cesarino, 2019; Han et al., 2022). All are detrimental to the health and well-being of plants which is why plants as immobile organisms have developed physiological responses to these stresses.

Rising global temperatures are expected to increase the occurrence of droughts (Balting et al., 2021; Ji et al., 2023). Forest trees are especially vulnerable to drought due to the sheer size of their water-conductive channels (Brodribb et al., 2020). Plants respond to water deficiency by closing their stomata to minimize water loss through evaporation, diminishing carbon fixation and hence growth (Brodribb et al., 2020). Long-term exposure to drought can ultimately lead to xylem embolism, which interferes with water transport (McDowell et al., 2008; Adams et al.,

2017; Choat et al., 2018; Brodribb et al., 2020). Due to the importance of lignin in providing waterproof and stability to water-conductive cells, its role in drought has gained interest. In *P. trichocarpa*, drought changed lignin composition but not the total lignin content (Hori et al., 2020). In *Eucalyptus*, drought had different effects on lignin content depending on the species and cambial age (Moura et al., 2011).

Global warming is also expected to increase the incidence of cold and freezing stress (Slater et al., 2021). The role of lignin in cold conditions remains inconclusive. An *Arabidopsis* mutant with reduced lignin content showed improved freezing tolerance (Ji et al., 2015). In *P. alba* × *P. glandulosa*, exposure to cold reduced lignin within a few days, likely due to reduced expression of several lignin-related *MYB* genes (Zhao et al., 2022b). In contrast, cold stress stimulated the expression of most ligninbiosynthetic genes except for the S-type lignin-related genes in *Eucalyptus gundal* (Ployet et al., 2018). As for drought stress, these results show that the relationship between lignin and cold/freezing tolerance remains unclear.

1.4.6 The role of lignin in biotic stress

Much like the organisms in the animal kingdom, plants are subjected to biotic stress caused by pathogens and pests. The primary protection against pathogens is the cuticle covering the outermost layer of the epidermis of organs such as leaves (Reina-Pinto & Yephremov, 2009; Serrano et al., 2014; Ziv et al., 2018). The bark in the stems has a similar role as the outermost protection (Campilho et al., 2020; Serra et al., 2022). The next layer of physical protection is the cell wall, which acts as the physical protection between the cell membrane and apoplast and is a critical component in pathogen recognition and signaling (Nühse, 2012;

Malinovsky et al., 2014; Miedes et al., 2014; Wan et al., 2021; Swaminathan et al., 2022).

Lignin plays a central role in plant defense. Lignin provides structural strength in the cell wall and physical support against pathogens (Lee et al., 2019). Accumulation of lignin in response to pathogen attacks has been shown in various species such as *Arabidopsis* (Lee et al., 2019), *P. tremuloides* (Bucciarelli et al., 2011) and *Picea abies* (Børja et al., 1995). Interestingly, biotic stress often specifically stimulates the biosynthesis is of the H-type lignin (Campbell & Ellise, 1992; Lange et al., 1995; Cabané et al., 2004; Cesarino, 2019). The reason for the H-type enrichment could be related to fewer biosynthetic steps and, hence, a smaller energy requirement for the biosynthesis of *p*-coumaryl alcohol compared to coniferyl or sinapyl alcohol. This type of lignin is often called as “stress” or “defense” lignin. The exact role of lignin has, however, remained somewhat unclear as lignin has been shown to both protect against (Ma et al., 2018; Lee et al., 2019; Yang et al., 2024; Ma, 2024) and predispose to pathogen attacks (Peltier et al., 2009; Funnell-Harris et al., 2010; Gallego-Giraldo et al., 2011). Also, the subunit content of lignin seems to influence the response to pathogens. Transgenic *Populus alba* × *tremula* trees with syringyl-rich lignin showed increased resistance towards wooddecaying fungi (Skyba et al., 2013) and tobacco plants likewise enriched in S-type lignin content showed improved resistance towards an oomycete and bacterial pathogen (Ma et al., 2018). These findings highlight the complicated relationship between lignin biosynthesis and plant immunity.

The phenylpropanoid pathway can diverge into the biosynthesis of compounds other than lignin, many of which are involved in plant defense

(Dixon et al., 2002; Yadav et al., 2020). For instance, coumarin accumulates in various species in response to pathogens and has been reported to possess antimicrobial qualities (Kai et al., 2006; Stringlis et al., 2019). Additionally, the presence of lignans and flavonoids stresses the broader role of the phenylpropanoid pathway in plant immunity beyond just lignin (Naoumkina et al., 2010).

1.5 Wood decaying fungi

Fungal decay of wood is essential to the nutrient and carbon cycles of forest ecosystems (Boddy & Watkinson, 1995). Fungi colonizing woody tissues has various roles in forest ecosystems and plant health. Saprotrophs colonize felled and dead wood and contribute to decomposition. Endophytes are non-pathogenic fungi that inhabit living tissues without being detrimental to the health of the trees. Pathogens include both disease-causing fungi introduced from the environment and commensal fungi that can become pathogenic under conditions of compromised host health (Huhndorf et al., 2004). Despite the strong barrier of the lignified cell walls in woody tissues and advanced physiological responses to pathogens, some microorganisms have evolved to bypass these deterrents. The fungi with wood decaying abilities are traditionally classified as brown, white, and soft rot (Goodell et al., 2008).

Members of the phylum of Basidiomycota, brown rot fungi, primarily attack cellulose and hemicellulose, retaining the oxidized lignin that gives the brown color of the wood (Illman, 1991; Goodell et al., 2003; Baldrian & Valásková, 2008; Goodell et al., 2008). Brown rot fungi are generally regarded as either conifer specialists or generalists (non-selective), as

evidenced by their ability to infect a wide range of species (Holb, 2008; Hu et al., 2011; Krah et al., 2018).

In contrast to brown rot fungi, most white rot fungi are angiosperm specialists and can break down both lignin and the carbohydrate backbones of cell walls (Goodell et al., 2008; Krah et al., 2018). White rots deploy various enzymes, including lignin peroxidase, manganese peroxidase and laccase, to break down lignin (Kirk & Cullen, 1998; Leonowicz et al., 1999; Presley et al., 2018), resulting in bleached, degraded wood (Goodell et al., 2008). *Phellinus tremulae* is a well-known white-rot fungus that infects aspen trees through wounds and branch stubs (Wikström, 1976), causing aspen trunk rot, also known as white trunk rot. Other white-rot fungi infecting *Populus* trees are *Armillaria* species causing root rot (Stanosz & Patton, 1987) and members of the *Trametes* genus (Leviu & Castro, 1998).

The third group of fungi are soft rots that most often belong to the phylum of Ascomycota (Goodell et al., 2008). Soft rots are divided based on their erosion type to type 1 and type 2. Type 1 soft rot forms cavities within the S2 layer of the secondary cell walls, while type 2 shows a similar erosion type to white rot fungi by causing a soft, spongy texture of the infected wood (Blanchette et al., 1990; Schwarze, 2007). They are also often found in conditions having high moisture content where brown or white rots do not thrive (Goodell et al., 2008). The soft-rot fungus *Cadophora* was also observed in decaying wooden structures in the Arctic (Blanchette et al., 2004) and in young aspen ramets (**Paper III).**

Forest trees are believed to be exposed to new pests and pathogens due to global warming (Budde et al., 2016; Naidoo et al., 2019). This will create a significant challenge for the forest industry and ecosystems. Due to the

economic damage caused by pathogens such as wood-decaying fungi, it is important to understand the pathogenesis and identify potential genetic variations in resistance to pathogens (Sniezko, 2006). Natural variation has been observed in trees' response to various pathogens such as ash dieback and white pine blister rust (Sniezko, 2006; Budde et al., 2016). This variation and current advancements in sequencing technology make it feasible to find molecular markers for pathogen resistance, that could be implemented in marker-assisted selection (Biselli et al., 2022). Research in various *Populus* species has recognized potential genetic markers associated with resistance to pathogens like *Melampsora* leaf rust (Stirling et al., 2001; La Mantia et al., 2013) and pests (Carletti et al., 2016; Zinkgraf et al., 2016; Newcombe et al., 2018). These earlier studies are encouraging and pave the way to find genetic markers for breeding of resistance towards new pathogens in forest trees.

2. Research aims

This PhD project aimed to understand the natural variation of diverse treerelated traits in Swedish aspen trees, understand the underlying genetics by combining multiple biological layers, and find potential markers associated with growth, saccharification, lignin content, and fungal resistance.

In **Paper I**, we focused on identifying traits that impact the bioconversion of aspen woody biomass. We also explored the genetic architecture of these traits and aimed to identify potential genetic markers for them.

In Paper II, we sought to identify key genes contributing to the natural variation of lignin content and composition in aspen wood. This study used transcriptomic data to elucidate the variation on a transcriptional level and understand which factors are involved in regulating lignin content.

Paper III aimed to identify the fungi colonizing woody tissues of aspen, explore resistance to the fungal infections, and determine the relationship between cell wall chemistry and fungal abundance.

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3. Materials and Methods

3.1 Swedish Aspen collection

Most of the data in my articles and thesis were collected from European aspen (commonly called as “aspen”) individuals that were part of the Swedish Aspen collection (SwAsp) grown in the common garden in Ekebo (13.1°E, 55.9°N) (Luquez et al., 2008). SwAsp has 113 individual genotypes originating from 12 different locations in Sweden. Genome sequencing and SNP calling of the SwAsp genotypes have been reported by Rendón-Anaya et al. (2021). This thesis used the latest version of aspen genome assembly v2.2 (Robinson et al., 2024).

The first harvest of SwAsp grown in Ekebo was in 2014 when stem discs were collected from felled 10-year-old aspen clones. Material from this harvest was presented in **Paper I**. New ramets grew from the harvested trees, which were harvested in 2019. Similarly, stem discs were collected for phenotyping. This material was used in **Paper** II & **III** (Figure 3).

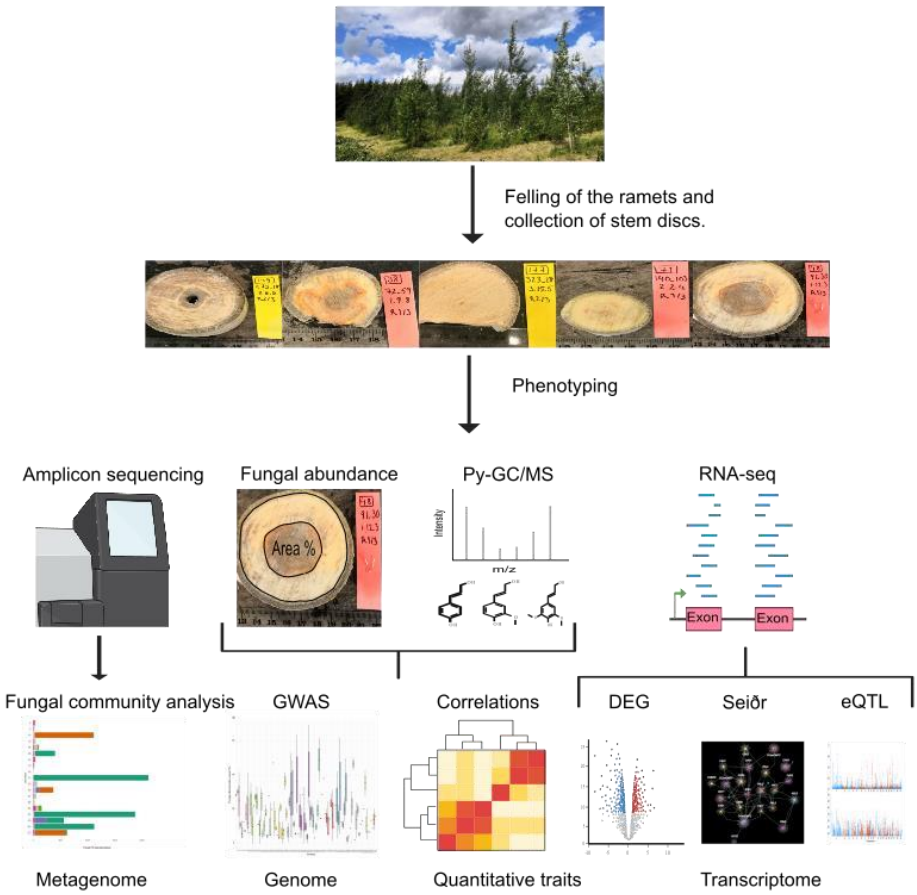

Figure 3. Overview of the experimental design for the 2019 harvest of the Swedish Aspen trees in Ekebo. Data were collected from several biological layers to investigate lignin biosynthesis and fungal infection of woody tissues.

3.2 Genome-wide association study (GWAS)

Genome-wide association study (GWAS) is a bioinformatic method used to identify associations between SNPs and traits within a population. The first GWAS investigations focused on understanding the genetic architecture of human diseases (Visscher et al., 2012). Since then,

advances in sequencing technologies and reductions in sequencing costs have led to a steady increase in the number of GWAS studies. The first plant GWAS was conducted in the model organism *Arabidopsis thaliana* (Aranzana et al., 2005), and numerous plant GWAS studies have followed (Tibbs Cortez et al., 2021). Within the Swedish aspen collection, GWAS has been utilized to study various traits (e.g. Grimberg et al., 2018; Mähler et al., 2020; Robinson et al., 2024; **Papers I-III**).

In our studies, GWAS was applied to identify genetic associations for various traits, including growth, xylem cell morphology, wood anatomy, saccharification, cell wall chemistry, and fungal abundance, utilizing the genome sequences of 99 SwAsp genotypes (Robinson et al., 2024). The single nucleotide polymorphisms (SNPs) identified in the SwAsp genotypes are available in Variant Call Format (VCF) format at the European Variation Archive (EVA, https://www.ebi.ac.uk/eva/). After quality filtering, 6,806,717 SNPs were identified, providing a dense marker set for genome-wide analysis.

Our studies used the Genome-wide Efficient Mixed Model Analysis (GEMMA) (Zhou & Stephens, 2012), which builds on the Efficient Mixed Model Association (EMMA) (Kang et al., 2008). GEMMA is approximately *n* times faster than EMMA, where *n* is the sample size, making it more suitable for genome-wide calculations in large datasets with dense SNP coverage (Zhou & Stephens, 2012). Unlike some approximation methods, GEMMA provides exact calculations for standard test statistics (e.g., Wald or likelihood ratio tests), reducing the risks of false positives and negatives. This enhances robustness and improves the detection of small-effect variants. GEMMA also supports the inclusion of covariates

(Zhou & Stephens, 2012). Our analyses used a genetic relatedness matrix as the primary covariate and the latitude of origin as a secondary covariate, although relatedness in the population was weak.

Sample preparation for GWAS began with removing outliers and normalizing non-normally distributed phenotypic data. Next, we estimated a best linear unbiased predictor (BLUP) for each trait. GEMMA was run utilizing the BLUP values for traits and the SNPs present in the SwAsp individuals (Robinson et al., 2024). The false discovery rate (FDR) was calculated according to the Benjamini-Hochberg procedure for each association to account for multiple testing and reduce the likelihood of false positives (Storey & Tibshirani, 2003).

3.3 Expression quantitative trait loci (eQTL)

Much like GWAS, expression quantitative trait loci (eQTL) analysis is used to find associations between SNPs and a trait of interest. In eQTL studies, the trait is gene expression (Nica & Dermitzakis, 2013). Understanding the role of SNPs in transcriptional regulation can uncover genomic hotspots that influence multiple transcripts or provide insights into how gene expression impacts a trait (Breitling et al., 2008; Gilad et al., 2008). It can also help determine the function of SNPs in non-coding regions of the genes identified in GWAS. SNPs identified as eQTLs are categorized as local (cis) or distant (trans) regulators. In **Paper II**, we used the threshold of 1Mbp to separate these two categories of eQTLs.

Our primary focus in **Paper II** was to investigate how lignin-biosynthetic genes are regulated in the developing xylem and to explore whether genomic hotspots exist in our data similar to findings reported by Balmant et al. (2020).

Much like GWAS, eQTL analyses commonly use linear or mixed-effect models. Our study used a linear model and applied confounder removal to reduce spurious noise in the data. Population structure, which arises partly from the genetic relatedness between individuals, is a critical factor in eQTL studies. Genetic relatedness can increase false-positive rates, preventing the detection of causal variants due to spurious associations that arise from shared ancestry unrelated to variation in a trait (Sul et al., 2018). However, we did not observe significant population structure in our dataset, consistent with findings from leaf transcriptome studies in the same SwAsp trees (Mähler et al., 2017). To account for population structure, the linear model included a principal component derived from a set of independent SNPs as a covariate, which helps to control the effects derived from genetic background. A similar approach was described by Mähler et al. (2017) using Matrix eQTL. Other factors, such as sampling time or sequencing batch effects, can create false positive associations. Implementing confounder removal reduced this noise and improved the quality of our data. Genes with expression variance below 0.5 were excluded to further refine the dataset.

Genome-wide eQTL analysis is computationally demanding, involving billions of SNP-gene pair evaluations. To address this challenge, we utilized Matrix eQTL, which performs much faster than most methods while allowing the inclusion of covariates. The Matrix eQTL algorithm achieves its efficiency through special preprocessing techniques and effectively handles computationally intensive tasks, such as large matrix operations. It supports separate analyses for local and distant eQTLs and calculates the False Discovery Rate (FDR) to address multiple testing issues.

3.4 Seiðr ensemble network

Gene co-expression networks can help to uncover biological processes within a cell or tissue and identify the specific genes driving a particular biological phenomenon. Gene expression patterns can help identify genes involved in specific biological pathways, which can also be used to annotate new genes based on their participation in a pathway. This approach has been used successfully, for example, in a study by Balmant et al. (2020) to identify critical regulators of lignin biosynthesis in *P. deltoides*. In **Paper II**, we used a gene co-expression network to investigate the regulation of lignification in the developing xylem of the SwAsp trees.

Our co-expression network was inferred using the Seiðr ensemble network tool, which was built upon the paradigm coined by Marbach et al. (2012) that an ensemble of inference methods surpasses the performance of any single predictor (Schiffthaler et al., 2023). Seiðr creates networks using multiple inference methods, which reduces bias and improves the network's overall performance. It is also highly scalable, making creating networks with tens of thousands of genes feasible. In **Paper II**, we used Seiðr to create a co-expression network of the developing xylem, which contained 34,623 expressed genes.

The network was inferred using RNA-seq data from 268 trees representing 99 SwAsp genotypes. Network inference was performed with thirteen algorithms implemented in the Seidr crowd network tool (v.0.14.4; Schiffthaler et al., 2023). Networks were combined using the inverse rank product approach (Zhong et al., 2014), and edges were pruned using a noise-corrected backbone method (Coscia & Neffke, 2017), and the clustering of the network was done using the INFOMAP algorithm (Rosvall & Bergstrom, 2008).

From the whole-transcriptome network, we inferred a lignin subnetwork of the 22 lignin-biosynthetic genes and their first neighbors to study the transcriptional regulation of lignin biosynthesis.

3.5 Amplicon sequencing

Amplicon sequencing of the internal transcribed spacer (ITS) region of ribosomal genes is widely used for mapping fungal communities in various samples (Schoch et al., 2012). This technique has been adapted to study both bacterial and fungal communities using specific sequence targets. The ITS region of ribosomal DNA (rDNA) is commonly used to detect bacteria (Frothingham & Wilson, 1993), while nuclear ribosomal DNA is used for fungi (Schoch et al., 2012; Kõljalg et al., 2013). This approach has been extensively applied to map the microbiomes of plant samples (Bálint et al., 2013; Bonito et al., 2014; Schneider et al., 2024). In **Paper III**, we used this method to identify potential fungal pathogens in woody tissue of 19 SwAsp trees representing 11 genotypes with varying disease symptoms.

PCR amplification followed the protocol of Beckers et al. (2016) using a two-step approach with primers gITS7 and ITS4, targeting the fungal ITS2 region of 18S rDNA. The ITS1 and ITS2 regions are the most commonly used target sequences for fungal identification, and in **Paper III**, we focused on amplifying the ITS2 region. ITS2 is highly variable and often species-specific, allowing rather accurate identification of the different fungal species (Bengtsson-Palme et al., 2013).

Fastq files were preprocessed primarily using dada2 (Callahan et al., 2016). Prefiltering steps included removing reads containing Ns and primers using cutadapt (Martin, 2011). Reads were then quality-filtered and trimmed. Filtered reads were subjected to error learning,

dereplication, denoising, and merging with up to one base mismatch, followed by chimera removal. ITS2 regions were retrieved from amplicon sequence variants (ASVs) using ITSx (Bengtsson-Palme et al., 2013) and clustered into operational taxonomic units (OTUs) via swarm (v2) (Mahé et al., 2015). Taxonomic classification was performed using dada2's naïve Bayesian classifier with the UNITE database (Abarenkov et al., 2024). Reads from other organisms besides fungi were removed.

3.6 Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

Total lignin content has been traditionally measured by the Klason (Klason, 1908; Schwanninger & Hinterstoisser, 2002) and thioacidolysis (Lapierre et al., 1985) method. However, these methods are timeconsuming and require large sample sizes. Our analysis employed instead a chromatography-based approach using pyrolysis-GC/MS (Gerber et al., 2012).

Pyrolysis involves heating the sample to a high temperature (450°C) to break down polymers in the ground material. The pyrolyzed compounds are then passed through capillary columns for separation. Subsequently, the separated compounds are ionized, and their mass-to-charge ratios are measured using mass spectrometry. The resulting chromatograms are analyzed with Multivariate Curve-Resolution by Alternate Regression (MCR-AR) to identify and quantify individual compounds (Gerber et al., 2012).

Cell wall components are determined based on their base peak mass, enabling the distinction of peaks into different compounds. The total lignin content is calculated by summing up the abundances of S, G, H, and P, with abundance determined for each peak (and compound) based on its

GC peak area as a percentage of the total GC peak area (Gerber et al., 2012).

It is important to adjust Py-GC/MS data according to the species analyzed. For instance in grass species, the abundance of *p*-coumarate and ferulate pyrolysis products may lead to overestimating G- and H-type lignin (del Rio et al., 2012). Similarly, H-type lignin is often overestimated, as its diagnostic pyrolytic products can also originate from polysaccharides and amino acids (Moldoveanu, 1998).

Despite these limitations, Py-GC/MS is an accurate, high-throughput method for estimating the relative quantities of cell wall compounds. It requires only a minimal amount of material for analysis. In our analyses, approximately 1 µg per sample was introduced into the pyrolyzer in two or three technical replicates.

3.7 Saccharification of lignocellulosic biomass

Saccharification, the enzymatic hydrolysis of lignocellulosic biomass, is a technique for extracting fermentable sugars from biomass, which can be further utilized, for example, for biomolecules or biofuels. However, enzymatic hydrolysis is inefficient due to various recalcitrants, such as lignin, and therefore biomass is pretreated to improve yield and efficiency. To understand this complex trait, we phenotyped the SwAsp trees for saccharification in **Paper I**, utilizing the technique presented by Gandla et al. (2015).

The woody tissue for the saccharification assays was ground and sieved to isolate particles between 0.1 and 0.5 mm in size. Two conditions were tested: untreated samples and samples pretreated with mild acidic treatment. The mild acidic pretreatment included 1% sulfuric acid catalyst

and microwaving the samples at 165°C for 10 minutes, followed by centrifugation to separate the solid and liquid fractions. The enzymatic hydrolysis of the pretreated solid fraction or the non-pretreated material composed of equal volumes of a cellulase preparation, and multifunctional enzyme derived from the fungus *Aspergillus niger*. The treated samples were incubated for 72 hours at 45°C (Gandla et al., 2015).

High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection was applied to quantify monosaccharides in processed samples. The sugar yields for each sample under various conditions were reported as grams per gram of sample dry weight (Gandla et al., 2015).

4. Results and Discussion

Genetic markers and tree properties predicting wood biorefining potential in aspen (*Populus tremula*) bioenergy feedstock (**Paper I**)

In this paper, we comprehensively analyzed natural variation in various stem traits and how these traits influence the biorefining of aspen biomass. *Populus* species are ideal tree model species for studying natural variation and the genetic background of complex traits (Jansson & Douglas, 2007), and large collections of poplars in species, such as *P. trichocarpa* and *P. nigra,* have been established in common gardens for these purposes (Faivre-Rampant et al., 2016; Chhetri et al., 2019). Even though natural variation in many traits, including wood properties, have been characterized in these species (Guerra et al., 2013; Porth et al., 2013; Yoo et al., 2017; Guerra et al., 2019), the phenotyping performed in this article is one of the most extensive.

We phenotyped 99 genotypes from the SwAsp collection for 65 traits. These traits covered different aspects such as anatomy, growth, wood chemistry, and saccharification. Various traits in SwAsp have been observed to display variation in SwAsp (Luquez et al., 2008; Robinson et al., 2012; Bernhardsson et al., 2013; Mähler et al., 2020), and the same was also observed in all our measured traits (**Paper I**, Fig.1a). Overall, the variation shown in these traits and the moderate broad-sense heritability indicate that many tree-related traits have the potential for genetic improvement.

Our second aim was to understand how these traits impact the recalcitrance of saccharification. Lignin content is one of the better-known recalcitrants of woody biomass (Studer et al., 2011; Yoo et al., 2017).

Various other biomass traits, such as cellulose crystallinity (Chang & Holtzapple, 2000; Zhao et al., 2012), degree of cellulose polymerization (Lu et al., 2019), and hemicellulose content and composition (Silveira et al., 2013; Auxenfans et al., 2017; Herbaut et al., 2018; Santos et al., 2018) also influence saccharification, which motivated us to explore the relationship between saccharification and our extensive phenotypic data. Orthogonal projections to latent structures (OPLS) (Trygg & Wold, 2002) analysis was utilized to study the relationship between all the traits and the essential measures of saccharification; the total wood glucose yield (TWG) and glucose release after pretreatment (GLUEHPT) (**Paper I**, Fig.4a-b). Although the model explained much of the variation in saccharification traits, it had relatively weak predictivity. However, the model supported the negative impact of lignin and hemicelluloses on saccharification efficiency, while several anatomical traits contributed positively to saccharification (**Paper I**, Fig.4c-d).

As a next step, we conducted a GWAS for every trait to find potential genetic markers for them. Results were filtered based on the less stringent threshold of FDR < 0.1. In total, 17 loci (or single nucleotide polymorphisms, SNP) had significance under the threshold of 0.1 FDR for five traits. Total wood glucose yield (TWG), reflecting the overall sugar yield in the whole woody biomass, associated with 12 loci, six of which were shared with the SNPs associated with stem height or diameter. The observed overlap between the two growth traits and TWG indicates a positive relationship between growth and glucose yields after saccharification. This was also evident as a high Pearson correlation between glucose yield with pretreatment and tree height and diameter (**Paper I**, Fig. 3). Genotypes 47 and 76, with exceptional stem height and diameter, also exhibited the highest TWG values. Both genotypes had a homozygous minor allele for the SNPs (chr10_2830421_T_G), located in

the exon of Potra2n10c20558 (E1 subunit of 2-oxoglutarate dehydrogenase, OGDH). The allelic variants for the chr10_2830421_T_G displayed significant differences in height, diameter, and TWG (**Paper I**, Fig.5h-j), indicating that the SNP chr10_2830421_T_G could act as a potent genetic marker for both growth and TWG.

Systems genetic analysis of lignin biosynthesis in *Populus tremula* (**Paper II**)

This part of the thesis explored the underlying genetics of lignin biosynthesis via a systems genetics approach in the SwAsp collection. Previous systems genetics studies have highlighted how perturbations of individual lignin-biosynthetic genes influence various phenotypes in *P. trichocarpa* (Wang et al., 2018). In another study by Xie et al. (2018), a systems genetics approach led to the discovery of a novel transcriptional regulator of lignification. In *P. deltoides*, a similar systems genetics approach identified a key regulator of the lignin-biosynthetic genes (Balmant et al., 2020). These findings motivated us to apply this same approach to aspen.

Given the lack of significant SNPs associated with cell wall chemistry in the GWAS analysis from **Paper I**, we adopted a more integrative multiomics approach to understand the genetic architecture of lignification better. RNA-seq data of developing xylem of 99 SwAsp genotypes were used to fill the knowledge gap of the previous study. We used the RNAseq to perform eQTL mapping to detect SNPs associating with gene expression. The analysis detected many eQTLs, showing extensive genetically controlled variation in gene expression of developing xylem tissues (**Paper II**, Fig.2c-d). We did not find evidence of distant-acting hotspots (**Paper II**, Fig. 2f), which is different from the eQTL analyses in other *Populus* species (Balmant et al., 2020; Yao et al., 2023; Zhang et

al., 2023b) but consistent with eQTL analysis in the leaf transcriptome of SwAsp (Mähler et al., 2017).

To further understand the transcriptomic regulation and coexpression network of the lignin-biosynthetic genes in the developing xylem of aspen, we assembled an ensemble network using Seiðr (Schiffthaler et al., 2023), which was then used to create a lignin subnetwork that consisted of the lignin-biosynthetic genes and their co-expressed genes. The ligninbiosynthetic genes in aspen were first identified based on sequence similarities with the previously reported *P. trichocarpa* lignin-biosynthetic genes (Shi et al., 2010). This resulted in the identification of 22 ligninbiosynthetic aspen genes (**Paper II**, Stable 3).

The lignin subnetwork showed that *CAD2* (Potra2n16c29966) was not coexpressed with the other network genes. We also observed that the expression of *CAD2* and *CCoAOMT3* (Potra2n8c17885) did not correlate with the rest of the biosynthetic genes. A high-resolution gene expression dataset in aspen wood (AspWood; Sundell et al., 2017) also showed a distinct expression pattern for *CCoAOMT3* and *CAD2* (**Paper II**, Fig.3a).

The lignin subnetwork allowed us to identify 89 transcription factors coexpressed with the lignin-biosynthetic genes. Among these transcription factors were homologs of Arabidopsis MYB42 and MYB85, which regulate the expression of lignin biosynthetic genes in Arabidopsis (Zhong et al., 2008; Geng et al., 2020). We also identified *HB5* (Homeobox protein 5; Potra2n1c1657; POPCORONA in *P. trichocarpa* Du et al., 2011), which was coexpressed with *PAL3* (Potra2n16c30091) (Figure 4). We validated the role of HB5 in lignification using hybrid aspen (*P. tremula* × *P. tremuloides)* RNAi lines. Two RNAi lines had significantly reduced G- and S-type lignin content (**Paper II**, Fig.4e). A binding motif for the *Arabidopsis*

homolog AtHB-15 was present upstream of the aspen *PAL3* gene, further supporting the role of HB5 in the regulation of *PAL3* (**Paper II**, Fig.7b).

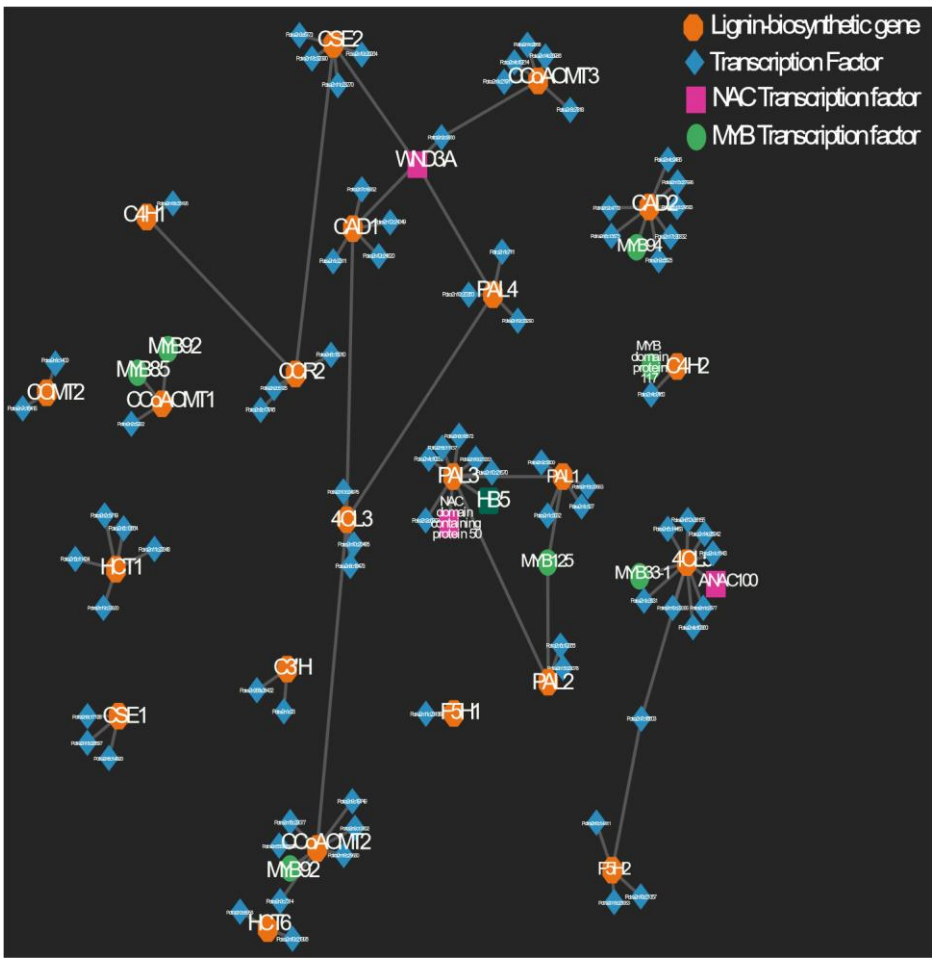

Figure 4. The MYB and NAC transcription factors identified in the lignin subnetwork in **Paper II.** The network illustrates co-expression patterns between lignin-biosynthetic genes (orange octagons), transcription factors (blue diamonds), MYB transcription factors (green circles), and NAC transcription factors (pink rectangles). Co-expression with the ligninbiosynthetic genes supports their role in regulation of the ligninbiosynthetic genes in the developing xylem of aspen ramets.

The lignin content was measured following the methods used in **Paper I,** and equivalent natural variation was observed in the lignin content of the 5-year-old ramets (**Paper II**, Fig.1 a-c). The levels of lignin and the lignin composition were comparable to other *Populus* species (Studer et al., 2011; Guerra et al., 2013; Porth et al., 2013; Muchero et al., 2015; Fahrenkrog et al., 2017b). Variation in the lignin content motivated us to perform GWAS for the cell wall chemistry data, which resulted in identification of a few genomic loci that associated with the variation in the S-type and G-type lignin content. The top SNP associated with the S-type lignin content was located in Chromosome 1 (**Paper II**, Fig.5a) in the coding region of a *UDP-glucosyl transferase family protein* (Potra2n1c2130). However, there was no expression of that gene in woody tissues in the AspWood database (Sundell et al., 2017) and it was therefore excluded from further studies. The second most significant SNP for S-type lignin was located in the exon of the *Galactosyl transferase family protein* Potra2n1c3762 (**Paper II**, Fig.5f), annotated as GT31_32 (Kumar et al., 2019). We did not observe differences in the expression of *GT31_32* between the genotypic groups having the different alleles of this SNP (**Paper II**, Fig. 5e). On the other hand, *GT31_32* was co-expressed with *CCoAOMT2*, which indicates the importance of transcriptional regulation of *GT31_32* in lignification. The whole-transcriptome network analysis revealed that GT31_32 is co-expressed with multiple homologs of the Arabidopsis Fasciclin-like arabinogalactan protein 12 (FLA12) (Geshi et al., 2013), which supports a role of for the aspen GT31_32 in glycosylation of arabinogalactan proteins.

The most significant associations were observed for the G-type lignin. The top SNP located upstream of *Ribose-5-phosphate isomerase-like* (RPIlike) gene (Potra2n11c22479). There was no clear allelic impact on the expression of the *RPI-like*, suggesting that the effect on G-type lignin

content is through another mechanism. A common challenge with GWAS results is the validation of the associations in independent populations or groups. We made efforts to validate our GWAS results in an independent aspen population (the UmAsp collection; Robinson et al., 2024). We observed significant differences in the G-type lignin content of the UmAsp individuals with different alleles for the significant SNP in the *RPI-like* gene, which confirms the role of RPI-like in the accumulation of the Gtype lignin in an independent aspen population (**Paper II**; Fig. S8b). The RPI-like is possibly associated with G-lignin content through its role in the pentose phosphate pathway (Howles et al., 2006; Stincone et al., 2015), an important pathway controlling carbon flux that eventually feeds also to cellulose and lignin biosynthesis. This association is further supported by evidence from *Arabidopsis*, where mutations in *Ribose-5-phosphate isomerases* (RPI1) led to a decrease in cellulose content (Howles et al., 2006). In contrast, a mutation in another pentose phosphate pathway gene, *transaldolase 2* (TRA2), led to decreased lignin content and altered S/G ratio (Vanholme et al., 2012b). Thus, it seems possible that RPI-like regulates G-type lignin accumulation through changes in the pentose phosphate pathway.

Abundant fungal infections in woody tissues of aspen ramets (**Paper III**)

Aspen is susceptible to many fungi that can cause wood discoloration. This part of the thesis studied discoloration due to fungal infections in the Swedish Aspen collection harvested in 2019 for **Paper II**. We observed discoloration and lesions in the stem discs during the harvest, most likely arising from fungal infection. The degree of the disease symptoms displayed a lot of variation within the population with the discolored stem area ranging from 0 to 69.2% of the wood surface area (**Paper III**, Fig.1b). This prompted us to analyze the identities of the fungal species that might

cause the wood discoloration and investigate whether the phenotypic variation is associated with genetic variation through a GWAS.

Similar to the resistance to other fungal infections (Muchero et al., 2018; Niemczyk & Thomas, 2020), the heritability was low ($H^2 = 0.22$) for the disease symptom phenotype (discolored wood area) in our study. The degree of the disease symptoms did not correlate with stem diameter, suggesting that the phenotype was not detrimental to the growth or that the infection was in such an early stage that it did not yet influence fitness.

Wood decay is best known to occur due to fungal pathogens (Goodell et al., 2008). Therefore, we performed amplicon sequencing of the fungal ribosomal DNA ITS2 region in woody tissues of 19 SwAsp ramets that showed varying degrees of wood discoloration. The amplicon sequencing data did not present evidence of major aspen pathogens, such as *Phellinus tremulae*. Instead, we found two fungal genera, *Cadophora* and *Ascocoryne*, to have significant representation in our samples (**Paper III,** Fig. 2a; Fig.3). *Cadophora* fungi have been recognized for their pathogenicity in various species (Sugar & Spotts, 1993; Frisullo, 2002; Prodi et al., 2008; Spadaro et al., 2011; Grantina-Ievina, 2015; Wenneker et al., 2016; Eichmeier et al., 2020; maral Carneiro et al., 2022; Kļaviņa et al., 2024; Tomada et al., 2024), but not in wood decay of *Populus* species. However, a member of the genus is involved in the wood decay of poplar-based wooden structures (Blanchette et al., 2004). *Ascocoryne* has not been reported to decay wood, but it has been found in stumps and logs of various angiosperm tree species, such as birch, aspen, and oa (indhe et al., 2004; eonhardt et al., 2019; Kļaviņa et al., 2024).

It is well known that cell wall chemistry impacts the resistance towards fungal pathogens. For instance, transgenic *P. alba x P. tremula* trees

enriched in S-type lignin exhibited increased resistance to three different brown- and white-rot fungi (Skyba et al., 2013). Lignin also accumulates in response to various other stress conditions (Cesarino, 2019). We found no major evidence for cell wall chemistry influencing fungal resistance in SwAsp as there was no clear correlation between the extent of wood discoloration and the Py-GC/MS traits, except for a positive one with the H-type lignin content (**Paper III**, Fig. 4). The H-type lignin is known to accumulate in various stress conditions (Lange et al., 1995; Cesarino, 2019), and our results suggest a role for H-type lignin also in connection to fungal infections in young aspen stems.

We performed a GWAS to identify potential markers for resistance against fungal infections in SwAsp. Although an increasing number of studies have utilized GWAS to map resistance markers (Bartoli & Rouz, 2017; Demirjian et al., 2023), only a few of them have been done in *Populus* species (Stirling et al., 2001; La Mantia et al., 2013; Muchero et al., 2018). We could not detect any associations for the extent of the fungal discoloration in the SwAsp population below the stringent (FDR<0.05) significance level. However, the top-ranked SNP was located in the gene region of Potra2n17c31796, a homolog of the *Arabidopsis Guanylatebinding protein-like 3* (GBPL3), and Potra2n17c31797, a homolog of the *Secondary wall-associated NAC domain protein 2* (SND2) (**Paper III**, Fig.5a-b). Interestingly, GBPL3 forms GBPL defense-activated biomolecular condensates (GDACs) that are formed in response to downy mildew (Huang et al., 2021). SND2 is better known as a master regulator of secondary cell wall formation (Hussey et al., 2011; Sakamoto & Mitsuda, 2015). Genotypic variants for this top-ranked SNP displayed significant differences in the abundance of the fungal discoloration (**Paper III**, Fig.5c). We also investigated the role of transcriptional regulation in fungal infections. A differentially expressed gene (DEG) analysis between

groups of SwAsp genotypes with extreme phenotypes identified Potra2n16c30219 as an interesting gene that differed significantly in expression between the phenotypic groups for both the fungal discoloration and the relative H-lignin content. The *Arabidopsis* homolog of Potra2n16c30219 is a lipid transfer protein (AT3G53980) associated with disease resistance in *Arabidopsis* (Hernández-Blanco et al., 2007). This supports the function of Potra2n16c30219 in fungal resistance in aspen, possibly in association with wood H-lignin accumulation.

5. Conclusions and future perspectives

In my thesis, I studied lignin and other complex tree traits in the Swedish aspen collection. Our studies showed that all these traits displayed natural variation, which we utilized to study the underlying genetic architecture by GWAS. In this thesis I also provide new insights into the transcriptional regulation and function of lignin, revealing lignin as a key recalcitrant of biomass conversion and having a versatile role in many other biological functions, including fungal pathogen resistance.

In paper I, our main aim was to understand how tree traits influence saccharification yield in aspen biomass. Lignin and some hemicellulose sugars were identified as the main recalcitrants, while the growth traits diameter and height positively impacted saccharification yield. Most traits exhibited variation and moderate heritability in the SwAsp collection, making them viable targets for the genetic improvement of feedstocks.

In our GWAS data we found a SNP in the exon of the E1 subunit of 2oxoglutarate dehydrogenase (E1-OGDH) associated with stem diameter, tree height, and total glucose yield, which makes it an attractive marker for growth and saccharification yield in aspen. Knocking out the OGDH gene by CRISPR-Cas9 could clarify the role of this gene. Two clones, SwAsp 47 and 76, had a homozygous minor allele for this SNP, and showed a significant increase in growth and TWG, attributes that make them commercially suitable for feedstock cultivation.

Our main aim in **Paper II** was to explore the genetic regulation of lignin biosynthesis by leveraging natural variation within the Swedish aspen (SwAsp) population. Lignin has multiple biological functions crucial to vascular plants' success. However, a more in-depth knowledge of the

genetic factors driving natural variation in its content and composition is still needed.

In Paper II, we used transcriptomic analyses in the woody tissue of the SwAsp trees to create a lignin subnetwork. The network revealed distinct expression patterns for *CCoAOMT3* and *CAD2*, also present in the AspWood database (Sundell et al., 2017). The network also revealed 89 transcription factors potentially regulating lignin biosynthesis. We validated the role of one of these genes through reverse genetics in hybrid aspen.

GWAS identified novel candidate genes associating with the G- and Slignin content, which contrasts with the lack of genetic associations with lignin content in **Paper I**, likely due to differences in tree age and growth conditions. A top-ranked SNP upstream of the RPI-like gene was associated with the G-type lignin and validated in another aspen population.

GT31_32, a putative glycosylase of the arabinogalactan proteins, displayed genetic polymorphism associated with the S-lignin content. *GT31_32* was co-expressed with *COMT2* and several *FLA* genes, suggesting its role in the interface between arabinogalactan proteins and lignin. Future work should validate the functionality of this protein in regulating S-lignin content using CRISPR-Cas9 or RNAi approach.

In Paper III, we explored molecular mechanisms underlying variation in fungal symptoms, identified as wood discoloration and decay, in SwAsp trees. The fungal symptoms correlated with H-type lignin content, suggesting a link between lignin composition and fungal susceptibility. Amplicon sequencing identified *Ascocoryne* and *Cadophora* as the dominant genera in affected trees, although their role as primary

pathogens remains uncertain. It is possible that the trees are first infected by bacterial pathogens, and amplicon sequencing of the bacterial 16S rDNA is needed to clarify this aspect. It is also unclear how the fungal pathogens infected the ramets, and detection of the extent of the fungal infections along the whole length of the stem is needed to identify the point of fungal entrance into the trees. Long-term follow-up of the remaining ramets are also needed to give insight into how malignant or benign the fungi are to the health of the trees. From GWAS, we identified a locus near GBPL3 and SND2; genes associated with defense and secondary cell wall formation. This locus may influence the tradeoff between growth and defense, and further studies on genotypic variants are needed to validate its role in fungal resistance.

In summary, we found significant variation in aspen in traits of economic interest, such as tree biomass production, wood chemistry and biomass conversion. We also provided new details on genetic factors contributing to variation in lignin content and composition as well fungal pathogen resistance in the Swedish aspen trees. Perhaps most importantly, our studies demonstrate that natural variation allows the identification of novel genetic markers for complex traits even in a moderately sized collection of individuals. These markers could be implemented in tree breeding programs. This will hopefully inspire further studies into complex traits in additional populations of aspen and other species.

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