

Lignin engineering in forest trees: From gene discovery to field trials

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ABSTRACT

Wood is an abundant and renewable feedstock for the production of pulp, fuels, and biobased materials. However, wood is recalcitrant toward deconstruction into cellulose and simple sugars, mainly because of the presence of lignin, an aromatic polymer that shields cell-wall polysaccharides. Hence, numerous research efforts have focused on engineering lignin amount and composition to improve wood processability. Here, we focus on results that have been obtained by engineering the lignin biosynthesis and branching pathways in forest trees to reduce cell-wall recalcitrance, including the introduction of exotic lignin monomers. In addition, we draw general conclusions from over 20 years of field trial research with trees engineered to produce less or altered lignin. We discuss possible causes and solutions for the yield penalty that is often associated with lignin engineering in trees. Finally, we discuss how conventional and new breeding strategies can be combined to develop elite clones with desired lignin properties. We conclude this review with priorities for the development of commercially relevant lignin-engineered trees.

Key words: lignin, genetic engineering, breeding, CRISPR, field trial, forest trees

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INTRODUCTION

Fossil resources are currently the main feedstock for energy and organic compound synthesis. However, considering the high carbon footprint of the petrochemical industry, the development of renewable and carbon-neutral feedstocks is a critical societal challenge (Vanholme et al., 2013b; Galán-Martín et al., 2021; Huang et al., 2021; Yang et al., 2021). Lignocellulosic biomass is the most abundant renewable carbon source on earth and therefore a promising candidate. Wood is an important source of lignocellulosic biomass. It is mainly composed of secondary cell walls that are primarily made from cellulose, hemicellulose, and lignin. Cellulose can be used in the pulp and paper industry or can, together with hemicellulose, be hydrolyzed into monosaccharides that can be further converted into high-value compounds such as liquid biofuels, building blocks for bioplastics, cosmetics, etc. (Bozell et al., 2007; Corma et al., 2007; Vanholme et al., 2013b; Isikgor and Becer, 2015; de Vries et al., 2021b). The additional valorization of lignin has recently been recognized as essential to enable the economic viability of lignocellulosic biorefining (Liao et al., 2020; Abu-Omar et al., 2021).

Lignin in wood of forest trees is predominantly built from monolignols that are biosynthesized by a series of enzymatic conversions starting from the amino acid phenylalanine (Figure 1) (Freudenberg,

1959; Boerjan et al., 2003; Vanholme et al., 2019). After their biosynthesis in the cytosol, monolignols are translocated to the cell wall (Pesquet et al., 2010, 2013; Derbyshire et al., 2015; Serk et al., 2015; Vermaas et al., 2019; Blaschek et al., 2020; Perkins et al., 2022). Notably, monolignols are translocated not only to the cell walls of the cells that produced them but also to those of neighboring cells (Hosokawa et al., 2001; Tokunaga et al., 2005; Pesquet et al., 2013; Smith et al., 2013, 2017; De Meester et al., 2018, 2021). In the cell wall, monolignols are oxidized by peroxidases (PERs) and/or laccases (LACs) and subsequently polymerized into the lignin polymer through radical coupling, thereby generating a range of chemical bonds such as aryl ether (β -O-4), resinol (β - β), phenylcoumaran (β -5), biphenyl ether (5-O-4), spirodienone (β -1), and dibenzodioxocin (5-5/4-O- β) (Berthet et al., 2011, 2012; Lu et al., 2013; Ralph et al., 2019; Tobimatsu and Schuetz, 2019). Lignin amount and composition vary greatly within and among plant species, tissues, cell types, and cell-wall layers, and are influenced by developmental and environmental factors (Vanholme et al., 2019). Hardwood (angiosperm) lignin is mainly built from the monolignols coniferyl, sinapyl, and

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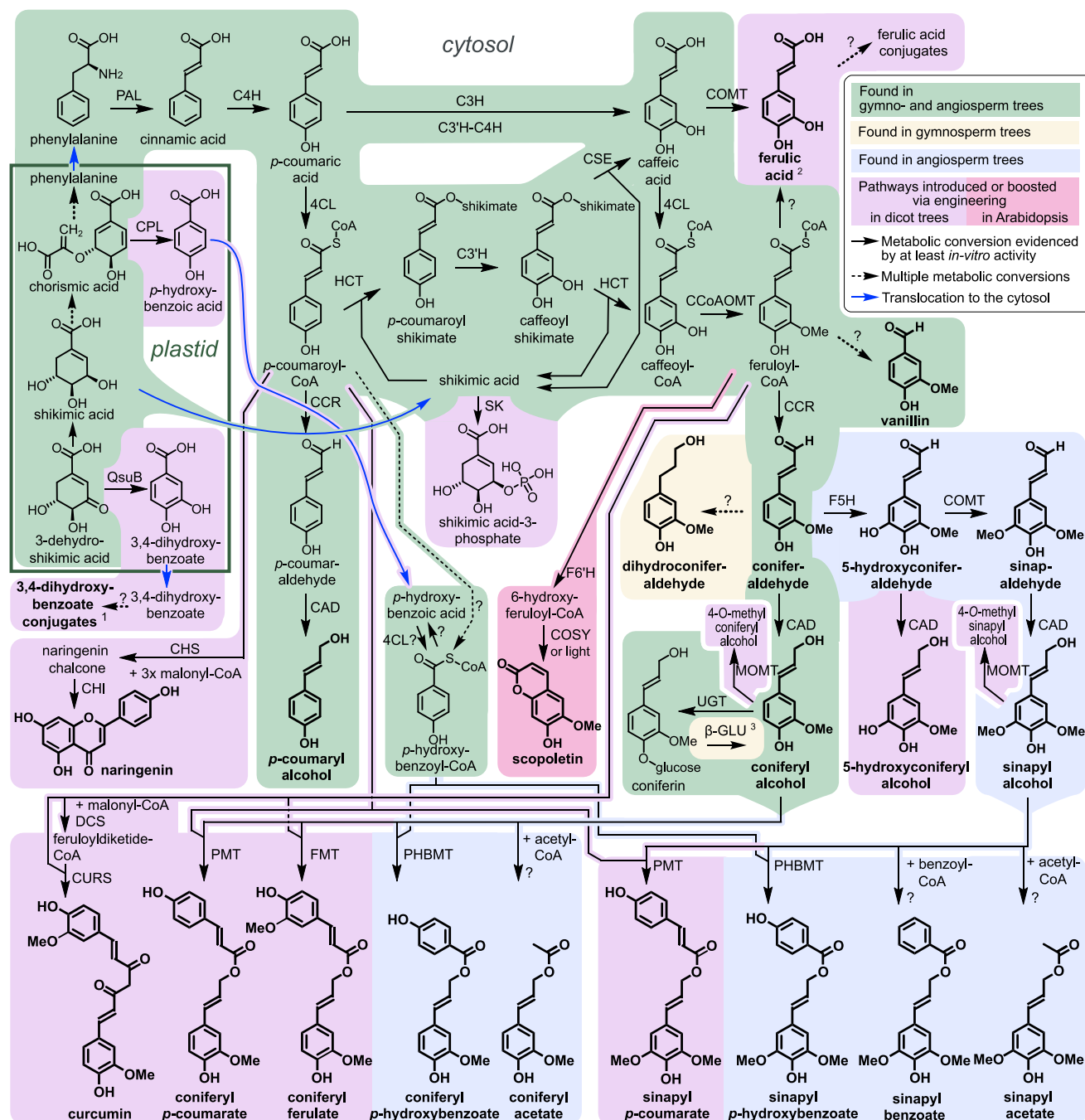


Figure 1. Metabolic pathways leading to lignin monomers.

Only the route toward lignin monomers is shown; side branches of the pathway that result in soluble metabolites are not shown, with the exception of ferulic acid conjugates (see footnote²). As enzymes may have different substrates and pathway perturbation may induce branching pathways, the figure includes only metabolic conversions for which strong evidence exists of involvement in biosynthesis of the shown end products. A question mark “?” means that the enzymes catalyzing the metabolic conversion(s) are not known. C3H, *p*-COUMARATE 3-HYDROXYLASE; CHI, CHALCONE ISOMERASE; other abbreviations can be found in the manuscript text. ¹The exact structures of the 4-dihydroxybenzoate conjugates are not known. ²Only a small portion of the ferulic acid pool is integrated into the lignin polymer; the majority of the pool is metabolized to soluble ferulic acid conjugates. ³The conversion of coniferin to coniferyl alcohol (which subsequently serves as a monomer for lignification) and glucose occurs in the apoplast of gymnosperm trees. Studies in *Ginkgo biloba*, *Pinus contorta*, *Picea abies* (Norway spruce), and *Chamaecyparis obtusa* (Japanese cypress) indicate that coniferin is a transport form of coniferyl alcohol in these species (Samuels et al., 2002; Tsuyama et al., 2013; Aoki et al., 2016; Väisänen et al., 2020).

(traces of) *p*-coumaryl alcohol, producing guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units, respectively. By contrast, softwood (gymnosperm) lignin is composed mostly of G units with a minor

fraction of H units. Depending on the plant species, other (less traditional) phenolic metabolites also act as monomers for lignification (Figure 1) (Ralph et al., 2019; Vanholme et al.,

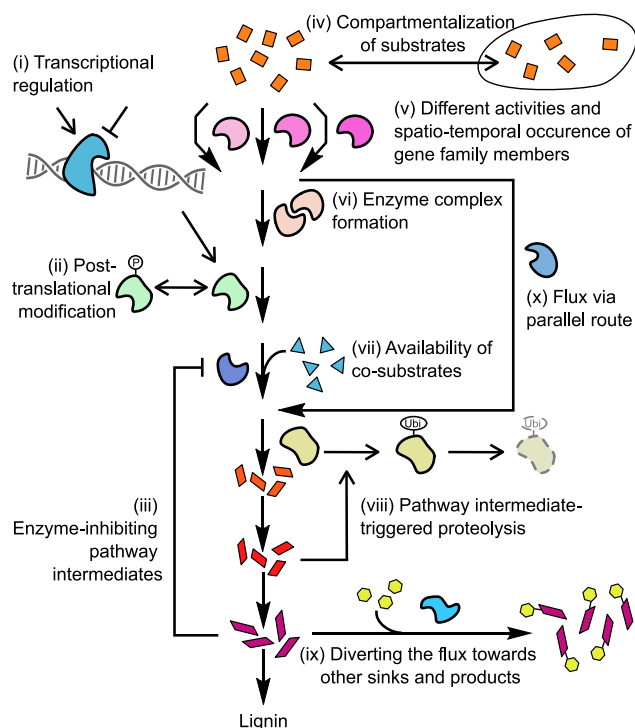


Figure 2. Schematic overview of multiple levels of lignin pathway regulation in trees.

Arrows with filled arrowheads represent metabolic conversions, and arrows with hollow arrowheads represent translocation, protein modification, or signaling. Pac-man-like spheres represent enzymes, and polygons represent metabolites.

2019; del Río et al., 2022). For instance, poplar lignin typically incorporates coniferyl acetate, sinapyl acetate, and sinapyl *p*-hydroxybenzoate, as well as traces of coniferyl *p*-hydroxybenzoate, sinapyl benzoate and coniferyl ferulate (Figure 1) (Morreel et al., 2004a, 2004b; Lu et al., 2004; Karlen et al., 2016; Zhao et al., 2021b; de Vries et al., 2022).

Currently, narrow profit margins limit the economic feasibility of employing fast-growing woody feedstocks, such as poplar, willow, and eucalyptus, as dedicated energy and specialty chemical crops at an industrial scale (Mahon and Mansfield, 2019). Because lignin is the main contributor to the recalcitrance of lignocellulosic biomass toward deconstruction, numerous research efforts have focused on altering lignin amount and/or composition, aiming to increase wood processing efficiency (Chanoca et al., 2019; Bryant et al., 2020). However, the *in planta* effects of pathway perturbations are often unpredictable because the flux through the lignin biosynthesis pathway is regulated at multiple levels (Figure 2): (i) at the transcriptional level (Zhong et al., 2010; Bonawitz et al., 2014; Ohtani and Demura, 2019); (ii) by post-translational protein modifications (Wang et al., 2015); (iii) by the abundance of enzyme-inhibiting pathway intermediates (Wang et al., 2014; Eudes et al., 2016; Yokoyama et al., 2021); (iv) by the compartmentalization of substrates (Widhalm et al., 2015; Eudes et al., 2016; Guo et al., 2018; Vanholme et al., 2019); (v) by the different activities and spatial expression patterns of gene family members (Li et al., 2015; Vanholme et al., 2019); (vi) by the formation of protein complexes (Chen et al., 2011; Widhalm

et al., 2015; Gou et al., 2018; Yan et al., 2019); (vii) by the availability of co-factors and -substrates (Tang et al., 2014; Vanholme et al., 2019; Hu et al., 2022); (viii) by pathway intermediate-triggered proteolysis of pathway enzymes (Guan et al., 2022); (ix) by branching pathways diverting the flux away from monolignols toward other sinks and products, such as flavonoids, benzenoids, phenylpropanoids, and monomer-glucosides (Vanholme et al., 2012); and (x) by the different parallel routes through which the pathway flux can flow via enzymes that often accept multiple substrates (Vanholme et al., 2019; Tsai et al., 2020). In addition, the outcome of the same pathway perturbation(s) in different species can differ significantly (Vanholme et al., 2019); e.g., *CAFFEYOYL SHIKIMATE ESTERASE* (CSE)-knockout *Arabidopsis*, *Medicago*, and poplar display huge differences in the frequency of H units incorporated into their lignin and the severity of the associated yield penalty (Vanholme et al., 2013c; Ha et al., 2016; de Vries et al., 2021a).

For poplar, a mathematical model was generated that estimates how changing the expression of pathway genes affects protein abundance, metabolite concentrations, metabolic flux, and various wood traits such as lignin amount and composition, biomass yield, and saccharification efficiency (Wang et al., 2018). Even so, this model also has its limitations; to generate the model, the analysis was restricted to one specific developmental stage, environmental condition, and poplar accession. Moreover, some factors that affect flux through the pathway, such as the formation of C3'H-C4H protein complexes, spatial distribution of substrates and enzymes (all xylary cell types were pooled to generate the model), and further metabolism of compounds (e.g., glycosylation), were not (yet) taken into account. It will therefore be crucial to assess the value of a lignin-engineering strategy and evaluate it under relevant forestry practices in economically relevant biomass germplasm such as (elite) poplar or eucalyptus clones.

In this review, we give an overview of the results obtained by engineering the levels of endogenous and exotic lignin building blocks in forest trees. In addition, based on data derived from greenhouse- and field-grown lignin-engineered trees, we discuss the link between lignin reduction and growth phenotypes on the one hand and between lignin reduction and the age of the tree and the environment in which it was grown on the other hand. We explore the possible causes of the frequently observed yield penalty in lignin-engineered trees and discuss strategies to avoid this yield penalty. We also address breeding strategies for the modification of lignin in forest trees, including traditional breeding with genomic selection and modern breeding techniques such as transgene expression and gene editing. We conclude this article with perspectives on the development and use of commercially relevant lignin-engineered trees.

ALTERING LIGNIN AMOUNT AND COMPOSITION VIA PATHWAY ENGINEERING

For industrial purposes aimed at the use of polysaccharides from wood (e.g., for pulping or for fermentable sugar-based biorefinery), a lower amount of lignin or lignin that is more easily extracted is a

desired trait. A reduction in lignin amount has typically been observed in trees with reduced activity of PHENYLALANINE AMMONIA LYASE (PAL), CINNAMATE 4-HYDROXYLASE (C4H), 4-COUMARATE:CoA LIGASE (4CL), *p*-COUMAROYLSHIKIMATE 3'-HYDROXYLASE (C3'H), *p*-HYDROXYCINNAMOYL-CoA:SHIKIMATE *p*-HYDROXYCINNAMOYLTRANSFERASE (HCT), CAFFEYOYL-CoA O-METHYLTRANSFERASE (CCoAOMT), and CINNAMOYL-CoA REDUCTASE (CCR) (Wang et al., 2018; reviewed in Chanoca et al., 2019). In these trees, lignin composition is often also affected. For instance, HCT- or C3'H-deficient trees deposit lignin with relatively large increases in the frequency of H units (Coleman et al., 2008; Vanholme et al., 2013a). Downregulation of CAFFEIC ACID O-METHYLTRANSFERASE (COMT) or CINNAMYL ALCOHOL DEHYDROGENASE (CAD) and overexpression of FERULATE 5-HYDROXYLASE (F5H) generally have less severe effects on lignin amount and mostly affect lignin composition. Reducing the efficiency of COMT and CAD boosts the incorporation of monomers that are typically present in trace amounts or below the detection limit in lignin of wild-type (WT) trees: 5-hydroxyconiferyl alcohol and 5-hydroxyconiferaldehyde are new units in COMT-deficient trees, whereas hydroxycinnamaldehydes are incorporated in higher amounts in CAD-deficient trees (Van Doorselaere et al., 1995; Baucher et al., 1996; Lapierre et al., 1999; Jouanin et al., 2000; Morreel et al., 2004a, 2004b; Van Acker et al., 2014; Yamamoto et al., 2020). The increased incorporation of these monomers into lignin is potentially desirable from an applied perspective because 5-hydroxyconiferyl alcohol gives rise to benzodioxane units that are anticipated to prevent covalent linkages between lignin and polysaccharide hydroxyl groups (Ralph et al., 2004), whereas incorporation of hydroxycinnamaldehydes gives rise to lignin that is more cleavable and soluble in alkaline conditions (Lapierre et al., 2004; Van Acker et al., 2017). Overexpression of F5H results in an increased fraction of S units (Franke et al., 2000; Stewart et al., 2009). Lignin with a high S/G ratio is interesting for both the pulp and paper industry and the lignin-first biorefinery because high-S lignin has a high fraction of beta-aryl ether bonds that are cleaved during alkaline pulping and via catalytic reductive fractionation, respectively (Baucher et al., 2003; Mansfield et al., 2012; Van den Bosch et al., 2015). For a thorough overview of lignin-engineering results achieved up to mid-2019 and from which the above-mentioned conclusions were drawn, we refer to Wang et al. (2018) and Chanoca et al. (2019) (Figure 1). Below, we focus on research published since then (Table 1; Figure 1).

CSE is a relatively newly discovered enzyme of the lignin biosynthetic pathway (Vanholme et al., 2013c; Ha et al., 2016; Saleme et al., 2017; Wang et al., 2021). Two independent studies investigated CRISPR-Cas9-induced mutations of CSE1 and CSE2 in poplar. One study found lignin and biomass yield reductions in *cse1 cse2* double mutants in *Populus tremula* × *Populus alba* cv 717-1B4 but no effects on lignin and growth in the respective single mutants (de Vries et al., 2021a). By contrast, the other study found that both *cse1* and *cse2* single mutants in *P. alba* × *P. tremula* var. *glandulosa* cv 84K showed up to 16% reduced lignin amount without biomass reduction (Jang et al., 2021). These different findings hint that the growth conditions or genetic background may interact with the *cse1* and *cse2* mutations, even though the two poplar genotypes used are interspecific hybrids of the same species.

Another recently discovered enzyme, *p*-HYDROXYBENZOYL-CoA MONOLIGNOL TRANSFERASE (PHBMT1), catalyzes the last step in the biosynthesis of coniferyl and sinapyl *p*-hydroxybenzoates in poplar (Zhao et al., 2021b; de Vries et al., 2022). Coniferyl and sinapyl *p*-hydroxybenzoates make up about 0.01% and 2.4%–6.5% of the lignin monomers in WT poplar, respectively, and might function in the regulation of the poplar gravitropic response (Regner et al., 2018; Kim et al., 2020; Zhao et al., 2021a). CRISPR-Cas9-induced knockout mutations in *PHBMT1* in poplar result in lignin with no detectable *p*-hydroxybenzoate decorations, whereas overexpression results in an increase in *p*-hydroxybenzoates, all without affecting biomass yield (Zhao et al., 2021b; de Vries et al., 2022). Lignin of *phbmt1* knockout poplar lines has a faster dissolution rate in acetyl bromide, whereas lignin dissolution of *PHBMT1* overexpression lines is slower than that of the WT (Zhao et al., 2021b). Also, the introduction of a bacterial *CHORISMATE PYRUVATE LYASE* (CPL) gene into poplar results in increased amounts of cell-wall-bound *p*-hydroxybenzoates. These plants have a reduction of up to 6% in lignin amount and a small yield penalty (Mottiar et al., 2022). The increase in *p*-hydroxybenzoate decorations on lignin is a potentially interesting trait, as saponification results in increased release of *p*-hydroxybenzoic acid that can be used in cosmetics, in polyester plastics, and as a precursor to a wide range of pharmaceutical compounds (de Vries et al., 2022).

In addition to genes involved in monolignol biosynthesis, genes involved in the polymerization of monolignols in the cell wall are also interesting targets for lignin pathway engineering. For example, overexpression of *LAC14*—which encodes a laccase enzyme with a high oxidizing efficiency toward coniferyl alcohol—in poplar results in 15% more lignin, a relative increase in G units, and decreased biomass yield compared with the WT (Qin et al., 2020). On the other hand, CRISPR-Cas9-induced mutation of *LAC14* in poplar results in lines with about 7% less lignin, a higher S/G ratio, and increased biomass yield (Qin et al., 2020).

Besides core genes of the lignin biosynthetic pathway, metabolic flux through the pathway can also be engineered via the depletion of essential co-factors. The expression of a bacterial *SHIKIMATE KINASE* (SK) gene in poplar results in a reduced shikimate pool in the cytoplasm, thereby hindering the HCT reaction for which shikimate is an essential substrate (Hu et al., 2022). The resulting transgenic poplars have a lignin content comparable to that of WT plants, but an increased amount of H units, a reduction in ether-linked G and S units, and a biomass yield penalty (Hu et al., 2022). The expression of another bacterial gene, *QsuB*, which encodes a 3-dehydroshikimate dehydratase, in poplar enables the plants to convert 3-dehydroshikimic acid into 3,4-dihydroxybenzoic acid (Unda et al., 2022). Transgenic poplars expressing *QsuB* partly divert the carbon flux away from the production of shikimate. These lines have up to 33% less lignin, incorporate ester-linked 3,4-dihydroxybenzoates in the lignin backbone, and have no biomass penalty.

Another strategy to divert carbon flux away from lignin biosynthesis has been achieved by expressing *MONOLIGNOL 4-O-METHYLTRANSFERASE 4* (*MOMT4*) in poplar (Cai et al., 2016). *MOMT4* is a methyltransferase engineered to methylate the

Species	Gene	Method	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	Reference
<i>P. trichocarpa</i>	<i>C4H1</i>	suppression via RNAi	↓13%	↓S/G	↑	n.d.	↓	Kim et al. (2020)
<i>P. trichocarpa</i>	<i>C3H3</i>	suppression via RNAi	↓42%	↑S/G	↑	n.d.	↓	Kim et al. (2020)
<i>P. trichocarpa</i>	<i>C4H1/ C4H2/ C3H3</i>	suppression via RNAi	↓50%	↑S/G	↑	n.d.	↓	Kim et al. (2020)
<i>Populus deltoides</i> × <i>Populus euramericana</i>	<i>4CL1</i>	fiber-specific suppression	↓21%	n.d.	n.d.	n.d.	WT	Cao et al. (2020)
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>4CL1</i>	vessel-specific suppression	↓26%	n.d.	n.d.	n.d.	WT	Cao et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	<i>4CL1</i>	CRISPR-Cas9	↓19%	↓S/G	WT	n.d.	WT	Tsai et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	<i>4CL5</i>	CRISPR-Cas9	WT	WT	n.d.	n.d.	WT	Tsai et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	<i>CSE1</i>	CRISPR-Cas9	WT	n.d.	n.d.	n.d.	WT	de Vries et al. (2021a)
<i>P. tremula</i> × <i>P. alba</i>	<i>CSE2</i>	CRISPR-Cas9	WT	n.d.	n.d.	n.d.	WT	de Vries et al. (2021a)
<i>P. tremula</i> × <i>P. alba</i>	<i>CSE1</i> and <i>CSE2</i>	CRISPR-Cas9	↓35%	↓S/G	↑	n.d.	↓	de Vries et al. (2021a)
<i>P. alba</i> × <i>P. glandulosa</i>	<i>CSE1</i>	CRISPR-Cas9	↓16%	n.d.	n.d.	n.d.	WT	Jang et al. (2021)
<i>P. alba</i> × <i>P. glandulosa</i>	<i>CSE2</i>	CRISPR-Cas9	↓16%	n.d.	n.d.	n.d.	WT	Jang et al. (2021)
<i>P. alba</i> × <i>P. glandulosa</i>	<i>HCT</i>	suppression via RNAi	↓11%	↓S/G	n.d.	n.d.	n.a.	Su et al. (2021)
<i>P. tremula</i> × <i>P. alba</i>	<i>CCR2</i>	CRISPR-Cas9	↓10%	WT S/G, incorporation of ferulic acid	↑	n.d.	WT	De Meester et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	<i>CCR2</i>	CRISPR-Cas9 knockout + vessel- and ray-specific overexpression of <i>AtCCR1</i>	↓18%	WT S/G, incorporation of ferulic acid	↑	n.d.	↓	De Meester et al. (2021)
<i>P. trichocarpa</i>	<i>CCR2</i>	suppression via RNAi	↓32%	↓S/G, incorporation of ferulic acid	n.d.	n.d.	↓	Yan et al., 2019
<i>P. tomentosa</i>	<i>mF5H2^a</i>	overexpression	n.d.	↑S/G	↑	n.d.	n.d.	Fan et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	<i>AtF5H1</i>	overexpression	WT	↑S/G	↑	n.d.	WT	Lapierre et al. (2021)
<i>P. trichocarpa</i>	<i>CAD1</i>	suppression via RNAi	↓9%	↑S/G, incorporation of aldehyde components	n.d.	n.d.	WT	Yan et al., 2019
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>LTF1</i>	fiber-specific suppression	↓43%	↓S/G	n.d.	n.d.	WT	Gui et al. (2020)
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>LTF1</i>	vessel-specific suppression	↓16%	↑S/G	n.d.	n.d.	↓	Gui et al. (2020)

Table 1. Overview of greenhouse-grown trees with modified expression of lignin biosynthesis genes published since mid-2019.

(Continued on next page)

Species	Gene	Method	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	Reference
<i>P. tremula</i> × <i>P. alba</i>	<i>CIDCS</i> and <i>CICURS2</i>	overexpression	↑23%	↓S/G	WT	n.d.	↓	De Meester et al. (2022a)
<i>P. alba</i> × <i>Populus grandidentata</i>	<i>MdCHS3</i>	overexpression	↓10%	WT S/G	↑	n.d.	WT	Mahon et al. (2022)
<i>P. tremula</i> × <i>P. alba</i>	<i>PHBMT1</i>	CRISPR-Cas9	WT	WT S/G, no detectable <i>p</i> -hydroxybenzoates	n.d.	n.d.	WT ^b	Zhao et al. (2021a); Zhao et al. (2021b)
<i>P. tremula</i> × <i>P. alba</i>	<i>PHBMT1</i>	overexpression	WT	WT S/G, increased <i>p</i> -hydroxybenzoates	n.d.	n.d.	WT	Zhao et al. (2021a); Zhao et al. (2021b)
<i>P. alba</i> × <i>P. grandidentata</i>	<i>PHBMT1</i>	overexpression	WT	WT S/G, increased <i>p</i> -hydroxybenzoates	n.d.	n.d.	WT	de Vries et al. (2022)
<i>P. alba</i> × <i>P. grandidentata</i>	<i>CPL</i>	overexpression	↓5%	↓S/G, increased <i>p</i> -hydroxybenzoates	↑	n.d.	↓	Mottiar et al. (2022)
<i>P. alba</i> × <i>P. glandulosa</i>	miR393	suppression via STTM	slightly ↑	n.d.	n.d.	n.d.	↑	Chu et al. (2021)
<i>P. tremula</i> × <i>Populus tremuloides</i>	<i>HpSK</i>	overexpression	WT	↑S/G, increased H units	↑	n.d.	↓	Hu et al. (2022)
<i>P. alba</i> × <i>P. grandidentata</i>	<i>QsuB</i>	overexpression	↓33%	↑S/G	↑	n.d.	WT	Unda et al. (2022)
<i>P. tremula</i> × <i>P. alba</i>	<i>BdPMT1</i>	overexpression	WT	WT S/G, incorporation of <i>p</i> -coumarates	↑	n.d.	WT	Lapierre et al. (2021)
<i>P. tremula</i> × <i>P. alba</i>	<i>AtF5H1</i> and <i>BdPMT1</i>	overexpression	WT	↑S/G, incorporation of <i>p</i> -coumarates	↑	n.d.	WT	Lapierre et al. (2021)
<i>P. trichocarpa</i>	<i>HSFB3-1</i>	CRISPR-Cas9	↓17%	n.d.	n.d.	n.d.	↑	Liu et al. (2021a)
<i>P. trichocarpa</i>	<i>MYB092</i>	CRISPR-Cas9	↓27%	n.d.	n.d.	n.d.	WT	Liu et al. (2021a)
<i>P. trichocarpa</i>	<i>HSFB3-1</i>	overexpression	↑10%	n.d.	n.d.	n.d.	↓	Liu et al. (2021a)
<i>P. trichocarpa</i>	<i>MYB092</i>	overexpression	↑18%	n.d.	n.d.	n.d.	↓	Liu et al. (2021a)
<i>P. tomentosa</i>	miR828	overexpression	↓13%	n.d.	n.d.	n.d.	↓	Wang et al. (2022)
<i>P. tomentosa</i>	miR828	suppression via STTM	↑15%	n.d.	n.d.	n.d.	↑	Wang et al. (2022)
<i>P. tomentosa</i>	<i>MYB171</i>	overexpression	↑12%	n.d.	n.d.	n.d.	↑	Wang et al. (2022)
<i>P. tomentosa</i>	miR6443	overexpression	WT	↓S/G	↓	n.d.	WT	Fan et al. (2020)
<i>P. tomentosa</i>	miR6443	suppression via STTM	WT	↑S/G	↑	n.d.	WT, lodging phenotype	Fan et al. (2020)
<i>Populus alba</i> × <i>P. glandulosa</i>	<i>MYB120</i>	dominant suppression via SDRX	↓59%	↓S/G	n.d.	n.d.	↓	Kim et al. (2021)
<i>P. deltoides</i>	<i>EPSPS</i> ^c	overexpression	ectopic lignification ^d	n.d.	n.d.	n.d.	n.d.	Xie et al. (2018)
<i>P. deltoides</i>	<i>EPSPS</i> ^c	suppression via RNAi	n.d.	n.d.	n.d.	n.d.	n.d.	Xie et al. (2018)
<i>P. tomentosa</i>	<i>LAC14</i>	overexpression	↑15%	↓S/G	n.d.	n.d.	↓	Qin et al. (2020)
<i>P. tomentosa</i>	<i>LAC14</i>	CRISPR-Cas9	↓7%	↑S/G	↑	n.d.	↑	Qin et al. (2020)

Table 1. Continued

For an overview of lignin-engineering results in greenhouse-grown trees achieved up to mid-2019, see Wang et al. (2018) and Chanoca et al. (2019). Since mid-2019, only lignin-engineering results from greenhouse-grown trees of *Populus* species have been published. n.d., not determined; S/G, syringyl/guaiacyl ratio; effect on total lignin is given as a percentage of the total lignin analyzed via different methods; see the corresponding reference for specific information. If multiple lines were tested, only the line with the most extreme change in lignin is shown. For abbreviations of gene names, see the manuscript text. RNAi, RNA interference; STTM, short tandem target mimic; SRDX refers to the protein domain LDLELR1; CRISPR, clustered regularly interspaced short palindromic repeats; At, *Arabidopsis thaliana*; Md, *Malus domestica*; Bd, *Brachypodium distachyon*; Cl, *Curcuma longa*.

^a*miF5H2* is an engineered miR6443-resistant form of *F5H2*.

^b*phbmt1* mutants showed a decrease in biomass until 2 months, when the plantlets were transferred from tissue culture to soil. When they grew for 3 or more months in soil, their growth appeared normal, although some mutant lines had a twisted trunk.

^cEPSPS is named EPSP in Xie et al. (2018) and EPSP-TF in Xie et al. (2020).

^dOverexpression under control of the CaMV35S promoter resulted in the ectopic deposition of lignin in cell walls of epidermis, phloem fiber, and pith cells as judged from microscopy sections stained with phloroglucinol-HCl. Lignin levels in the xylem were not quantified.

para-hydroxylic function of lignin monomeric precursors, preventing the derived methylated products from participating in the lignin coupling process. Compared with the WT, *MOMT4*-expressing lines display a 13% reduction in lignin content, a 75% reduction in S/G ratio, and a 50% reduction in *p*-hydroxybenzoates (predominantly acylating S units) in the cell wall, without affecting growth and biomass yield.

Another strategy for steering metabolic flux in the lignin biosynthesis pathway is via transcriptional regulation. 5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE (EPSPS) is a key enzyme in the shikimate biosynthetic pathway and has recently been identified as a transcriptional regulator of the phenylpropanoid pathway in poplar (Xie et al., 2018, 2020; Yao et al., 2021). The transcriptional regulatory functionality of EPSPS is defined by a single-nucleotide polymorphism (SNP), and natural poplar variants carrying this SNP have reduced lignin content (the amount of lignin reduction and the biomass yield were not reported) (Xie et al., 2018, 2020). Another example in which an allelic form of a transcription factor results in a reduced lignin content is the expression of a phosphorylation-null mutant of LIGNIN BIOSYNTHESIS-ASSOCIATED TRANSCRIPTION FACTOR 1 (LTF1) in poplar (Gui et al., 2019, 2020). LTF1 is a MYB transcription factor that functions as a transcriptional repressor of lignin biosynthesis in its unphosphorylated state. When LTF1 becomes phosphorylated in response to external stimuli, it undergoes degradation via the proteasome, resulting in the activation of lignification. Transgenic lines with constitutive expression of the *LTF1* phosphorylation-null mutant (*LTF1^{AA}*) display a decrease of up to 56% in lignin content but also a decrease in plant height (Gui et al., 2019, 2020), whereas expression of *LTF1^{AA}* under the control of a fiber-specific promoter results in a decrease of up to 43% in lignin content and a plant height similar to that of controls (Gui et al., 2020). MYB120 is a transcription factor involved in the activation of lignin and anthocyanin biosynthesis. Transgenic poplars with dominant suppression of *PtrMYB120* showed up to 59% less lignin, reduced anthocyanin levels, and a lower biomass yield (Kim et al., 2021).

Transcript levels can be post-transcriptionally regulated via microRNAs. MicroRNA393 (miR393) is a conserved miRNA family in plants that can regulate root growth, plant architecture, leaf development, and stress resistance (Chu et al., 2021). Poplar lines in which the miR393 family was blocked via short tandem target mimic (STTM) show an increased growth compared with WT trees, as well as a higher expression of lignin biosynthetic genes and a higher stem lignin content. How miR393 regulates lignin biosynthetic genes remains unknown (Chu et al., 2021). Overexpression of another microRNA, miR828, in poplar results in a reduced lignin content (−13%) in wood and a decreased biomass yield (Wang et al., 2022). Conversely, suppression of miR828 in poplar via STTM increases lignin content (+15%) and biomass yield (Wang et al., 2022). In addition, overexpression of *MYB171*, one of the direct targets of miR828, results in higher lignin amounts (+12%) and taller trees (Wang et al., 2022). Overexpression of miR6443, a microRNA that post-transcriptionally regulates *F5H2*, in poplar results in decreased transcript levels of *F5H2* and consequently a 36% reduction in S units together with normal growth (Fan et al., 2020). On the other hand, blocking miR6443 via STTM results in poplar with

increased *F5H2* transcript levels, lignin with a 122% increase in S units, and a lodging phenotype (Fan et al., 2020).

Of special interest from a biorefinery perspective is an increase in the amount of tension wood in angiosperm trees. Tension wood is a kind of reaction wood that is typically formed on the upper side of tilted stems or branches and has a high cellulose and low lignin content compared with normal wood (Sawada et al., 2018). *PtrHSFB3-1* and *PtrMYB092* have been identified as repressors of tension wood formation in poplar (Liu et al., 2021a). CRISPR-Cas9-induced mutations in *PtrHSFB3-1* or *PtrMYB092* result in a stem wood composition resembling that of tension wood (i.e., high cellulose and low lignin), whereas overexpression of *PtrHSFB3-1* or *PtrMYB092* in poplar results in low cellulose and high lignin contents (Liu et al., 2021a). *PtrHSFB3-1*-knockout poplars are about 9% taller than the WT, whereas the height of *PtrMYB092*-knockout poplars is similar to that of the WT. On the other hand, overexpression of *PtrHSFB3-1* or *PtrMYB092* results in a 10% or 67% reduction in plant height compared with WT poplar, respectively.

Most of the engineering strategies mentioned above that resulted in wood with reduced lignin amounts also resulted in a higher saccharification yield (in cases where saccharification was performed; Table 1). This is in line with the general finding that lignin amount is negatively correlated with saccharification and pulping yield (Baucher et al., 2003; Chen and Dixon, 2007; Van Acker et al., 2013). The notable exceptions are the CRISPR-Cas9-generated *4cl1* poplar mutants that, despite having a 19% reduction in lignin amount, do not show a significantly increased saccharification yield (Tsai et al., 2020). The authors suggested that the expected gain in saccharification yield from the reduced lignin amount was offset by changes in lignin structure in *4cl1* poplar mutants (Tsai et al., 2020). Interestingly, an altered xylem coloration (brown, red, pink, or orange) has frequently been observed in lignin-engineered trees, such as those with reduced expression of *4CL* (Wagner et al., 2009; Voelker et al., 2010; Zhou et al., 2015), *CCoAOMT* (Meyermans et al., 2000; Zhong et al., 2000; Jing et al., 2004), *CCR* (Leplé et al., 2007; Van Acker et al., 2014; De Meester et al., 2020), *COMT* (Van Doorselaere et al., 1995; Tsai et al., 1998; Jouanin et al., 2000; Pilate et al., 2002), and *CAD* (Baucher et al., 1996; MacKay et al., 1997; Pilate et al., 2002; Van Acker et al., 2017; Yamamoto et al., 2020). In *CCR*-deficient wood, the reddish coloration has been hypothesized to be caused by the incorporation of ferulic acid, the de-esterification product derived from the *CCR* substrate feruloyl-coenzyme A (CoA), into the lignin polymer; synthetic lignins made from coniferyl alcohol, sinapyl alcohol, and ferulic acid are reddish (Leplé et al., 2007; Ralph et al., 2008). In *CAD*-deficient wood, the red xylem coloration is potentially caused by the formation of S'(8-S)' (or β - β' di-sinapyl aldehyde), a reddish dimeric coupling product made from sinapaldehyde, one of the substrates of *CAD* (Fournand et al., 2003).

EXOTIC LIGNIN

Basically, any molecule with a phenolic function that can be oxidized via the activity of laccases and/or peroxidases can serve as a lignin monomer, on the condition that the molecule can be translocated to the cell wall (Ralph, 2006; Mottiar et al., 2016;

del Río et al., 2020). Although it has been suggested that ATP-binding cassette (ABC) transporter family proteins could be involved in the transport of monomers across the plasma membrane (reviewed in Perkins et al., 2019), more recent molecular dynamics simulations and experimental observations support a passive permeation mechanism for monomers and dimers across the plasma membrane lipid bilayer (Vermaas et al., 2019; Perkins et al., 2022). However, carboxylated or glycosylated phenolic monomers have been predicted to not diffuse easily through the plasma membrane and to require transporters for translocation across the membrane (Vermaas et al., 2019). These predictions are in agreement with experimental observations. For instance, only minor levels of ferulic acid are integrated into the lignin of *Arabidopsis* and poplar with compromised *CCR* activity, whereas soluble ferulic acid conjugates accumulate to high levels in these plants (Figure 1) (Leplé et al., 2007; Ralph et al., 2008; Van Acker et al., 2014; De Meester et al., 2018, 2020). The apparent absence of a need for specific transporters for non-glycosylated and non-carboxylated monomers (Vermaas et al., 2019; Perkins et al., 2022) suggests possibilities for the incorporation of non-canonical monomers that are normally not part of—or are present at very low levels in—the lignin polymer, through the introduction of novel metabolic routes (Figure 1).

An increasing number of studies demonstrate that lignin can be engineered through the coupling of non-canonical monomers (Figure 1). A first example of such a non-canonical monomer is coniferyl ferulate, a specialized metabolite that accumulates in *Angelica sinensis* but is naturally only found at trace levels in lignin (Karlen et al., 2016). When *FERULOYL-COA:MONOLIGNOL TRANSFERASE (FMT)* from *A. sinensis* is expressed in poplar, coniferyl ferulate copolymerizes with natural lignin monomers in the cell wall (Wilkerson et al., 2014). With the integration of coniferyl ferulate, ester linkages are introduced into the lignin backbone, rendering the lignin more easily extractable in alkaline conditions (Wilkerson et al., 2014). Second, curcumin is a soluble metabolite in *Curcuma longa* (turmeric) that is not known to be part of the natural lignin of tree species. By overexpression of two genes from turmeric, *DIKETIDE-CoA SYNTHASE (DCS)* and *CURCUMIN SYNTHASE (CURS)*, curcumin has been successfully biosynthesized and integrated into the cell walls of *Arabidopsis* and poplar (Oyarce et al., 2019; De Meester et al., 2022a). However, in contrast to transgenic *Arabidopsis* plants, transgenic poplars have 23% more lignin, an altered lignin composition that resembles stress lignin, no increase in saccharification efficiency after alkaline pretreatment, and a yield penalty. These data highlight the importance of translating research from model organisms into crops. Third, the flavonoid tricetin is naturally present in lignin of grasses (Lan et al., 2016), but flavonoids have not yet been naturally found in the lignin of tree species. However, the expression of *CHALCONE SYNTHASE (CHS)* from *Malus domestica* (apple) in poplar results in incorporation of the flavonoid naringenin into the lignin polymer, reduced total lignin content, and increased cell-wall carbohydrate content, with no effect on biomass yield (Mahon et al., 2022). Wood from these *CHS*-overexpressing poplars is substantially easier to saccharify after no pretreatment or dilute acid pretreatment (Mahon et al., 2022). Fourth, sinapyl *p*-coumarate and, to a lesser extent, coniferyl *p*-coumarate are common natural

	Biomass (g)	Lignin amount (%)	Lignin composition (S/G ratio)
WT (greenhouse)	n.a.	22.0 ± n.a.	2.5 ± n.a.
WT (field, Piedmont site)	93.8 ± 9.2	26.4 ± 0.4	1.7 ± 0.1
WT (field, coastal plain site)	136.1 ± 14.0	25.5 ± 0.5	1.9 ± 0.1
WT (field, mountain site)	1088.9 ± 88.5	23.4 ± 0.3	2.0 ± 0.0

Table 2. Biomass, lignin amount, and lignin composition of WT *P. trichocarpa* trees grown in the greenhouse and at 3 different field locations in North Carolina (USA).

In the field, the trees were planted in April 2009, coppiced for the first time in January 2010, and coppiced for the second time in January 2011. Values (means ± standard error) are given for plant material derived from the greenhouse or the second coppice in the field. n.a., not available.

Source: Stout et al. (2014).

monomers of grass lignin (Grabber et al., 1996) but are absent or close to the detection limit in tree species. Overexpression of *BdPMT1*, a *Brachypodium distachyon* gene that encodes a *p*-COUMAROYL-CoA MONOLIGNOL TRANSFERASE, in poplar results in lignin with an even higher frequency of *p*-coumarates compared with *Brachypodium*, without affecting growth (Lapierre et al., 2021). The lignin of the transgenic trees has a lower polymerization degree and an improved solubility in cold NaOH solution, resulting in an improved saccharification yield of alkali-pretreated wood (Lapierre et al., 2021). The *p*-coumaroylation of lignin could not be further boosted by stacking the overexpression of *BdPMT1* with the overexpression of *F5H1* from *Arabidopsis*, even though the stacked lines had a higher frequency of syringyl units and the *p*-coumaroylation of sinapyl alcohols is favored (Lapierre et al., 2021).

Examples of alternative lignin monomers that have been tested in non-tree species are disinpoyl glucose and the coumarin scopoletin. Because disinpoyl glucose has two ester bonds, its incorporation into the lignin polymer could potentially result in a weaker backbone and easier degradability in alkaline conditions. Engineering of the phenylpropanoid pathway in *Arabidopsis* resulted in the successful accumulation of disinpoyl glucose (Lee et al., 2017). However, in line with the low efficiency of passive diffusion of glycosylated monomers into the cell wall (Vermaas et al., 2019), disinpoyl glucose was not integrated into the lignin of the transgenic plants (Lee et al., 2017). In two different studies, *Arabidopsis* has been engineered to boost scopoletin production by overexpressing *FERULOYL-CoA 6'-HYDROXYLASE (F6'H)* alone or together with *COUMARIN SYNTHASE (COSY)* (Sakamoto et al., 2020; Hoengenaert et al., 2022). Introduction of scopoletin into lignin theoretically introduces ethers that make it more cleavable than WT lignin. Introduction of the metabolic route to scopoletin results in the successful incorporation of scopoletin into lignin and improved saccharification after alkaline pretreatment, making this a promising strategy for translation into trees (Hoengenaert et al., 2022).

Specific laccases may be required to allow the coupling of certain exotic monomers into the lignin polymer. It has been shown that laccase ChLAC4 catalyzes the oxidation of coniferyl alcohol and its subsequent coupling into lignin in the seed coat of *Cleome hassleriana* and that ChLAC4 does not efficiently oxidize caffeoyl alcohol (Zhuo et al., 2022). Instead, ChLAC8 is responsible for oxidation of caffeoyl alcohol and its coupling into the lignin of *C. hassleriana* seed coats (Zhuo et al., 2022). These findings

suggest that ChLAC8 could be essential in lignin-engineering strategies aimed at introducing caffeoyl alcohol into the lignin of trees and also that laccases might be engineered to enable or facilitate polymerization of specific exotic monomers.

LESSONS LEARNED FROM OVER 20 YEARS OF FIELD TRIALS WITH LIGNIN-ENGINEERED TREES

For practical and regulatory reasons, most studies on lignin engineering have been performed with greenhouse-grown trees (see above and Table 1). However, unlike greenhouse-grown trees, field-grown trees undergo annual seasonal cycles of growth and dormancy and interact with a range of biotic and abiotic environmental factors, such as field location, wind, drought, and pathogens. These factors greatly influence the cell-wall composition and growth of plants (as illustrated in Table 2 for WT trees and described below for lignin-engineered trees) (Cesarino, 2019). Because field trials also allow the trees to be evaluated under relevant agricultural practices (such as short-rotation coppice culture), they are a crucial step in translating fundamental knowledge generated in the greenhouse to conditions closer to industrial exploitation (Pilate et al., 2015).

Table 3 summarizes reports on all field trials performed with lignin-engineered trees. To date, eucalyptus trees downregulated in *C3'H* or *C4H* and poplar trees downregulated in *4CL*, *CSE*, *CCoAOMT*, *CCR*, *COMT*, *CAD*, or *LTF1* have been evaluated in the field. Of particular interest are studies that examined the performance of the very same transgenic line in both the greenhouse and the field, preferably planted in multiple contrasting locations and harvested over multiple rotations. Data from these studies lead to the following general observations:

- (1) The lignin reduction and growth phenotype of trees engineered to produce less lignin are highly dependent on the growth environment (genotype × environment interactions). Indeed, the very same transgenic line can behave significantly differently depending on whether it is grown in the greenhouse or the field. For example, the reduction in lignin in *4CL*-, *CCoAOMT*-, *CCR*-, or *CAD*-downregulated poplars was greater when the trees were grown in the greenhouse than when they were grown in the field (Wang et al., 2012; Stout et al., 2014; Van Acker et al., 2014, 2017; Xiang et al., 2017; De Meester et al., 2022a). Moreover, although *CCR*-, *CAD*-, and vessel-specific *4CL*-downregulated poplars grew normally in the greenhouse, the same lines displayed a yield penalty when grown

Species	Gene	Method	Tested in GH?	Location	# Lines	Field size	Field duration	Age of the stems at harvest	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	References
<i>E. urophylla</i> × <i>E. grandis</i>	<i>C4H</i>	RNAi	no	FL, USA	9	n.a.	2 years	2 years	↓40% to ↓30%	↓S/G	↑	n.d.	↓	Sykes et al. (2015)
<i>E. urophylla</i> × <i>E. grandis</i>	<i>C3'H</i>	RNAi	no	FL, USA	6	n.a.	2 years	2 years	↓28% to ↓25%	↓S/G	↑	n.d.	↓	Sykes et al. (2015)
<i>P. tremula</i> × <i>P. alba</i>	<i>4CL</i>	antisense	no	OR, USA	14	10–15 plants per line	2 years	2 years	↓10% to WT	↓S/G to ↑S/G	WT	n.d.	↓	Voelker et al. (2010)
<i>P. tomentosa</i> Carr.	<i>4CL</i>	sense and antisense	no	Hebei, China	5	60 plants per line	6 years	1 year	↓28% to ↓5%	↑S/G	n.d.	n.d.	↑	Tian et al. (2013)
<i>P. nigra</i> × <i>Populus maximowiczii</i>	<i>4CL</i>	antisense	yes	mountain, NC, USA	3	n.a.	3 years	2 years	↓8% to WT [↓47% to ↓8%]	↑S/V [↑S/V]	↑ [n.d.]	n.d. [n.d.]	n.d. [n.d.]	Xiang et al. (2017) [Xiang et al., 2017]
<i>P. nigra</i> × <i>P. maximowiczii</i>	<i>4CL</i>	antisense	yes	mountain, NC, USA	3	n.a.	3 years	3 years	WT [↓47% to ↓8%]	↓S/V to ↑S/V [↑S/V]	↑ [n.d.]	n.d. [n.d.]	n.d. [n.d.]	Xiang et al. (2017) [Xiang et al., 2017]
<i>P. nigra</i> × <i>P. maximowiczii</i>	<i>4CL</i>	antisense	yes	coastal plain, NC, USA	3	n.a.	3 years	2 years	↓7% to WT [↓47% to ↓8%]	↓S/V to ↑S/V [↑S/V]	WT [n.d.]	n.d. [n.d.]	n.d. [n.d.]	Xiang et al. (2017) [Xiang et al., 2017]
<i>P. nigra</i> × <i>P. maximowiczii</i>	<i>4CL</i>	antisense	yes	coastal plain, NC, USA	3	n.a.	3 years	3 years	↓9% to WT [↓47% to ↓8%]	↑S/V [↑S/V]	↑ [n.d.]	n.d. [n.d.]	n.d. [n.d.]	Xiang et al. (2017) [Xiang et al., 2017]
<i>P. trichocarpa</i>	<i>4CL</i>	antisense	yes	coastal plain, NC, USA	12	149 plants per line	2 years	1 year (2 y/o roots)	↓37% to WT [↓46% to ↓5%]	↓S/G to ↑S/G [↓S/G and ↑S/G]	n.d. [n.d.]	n.d. [n.d.]	↓90% to ↑83% [n.d.]	Stout et al. (2014) [Stout et al., 2014]
<i>P. trichocarpa</i>	<i>4CL</i>	antisense	yes	Piedmont, NC, USA	12	149 plants per line	2 years	1 year (2 y/o roots)	↓33% to WT [↓46% to ↓5%]	↓S/G to ↑S/G [↓S/G and ↑S/G]	n.d. [n.d.]	n.d. [n.d.]	↓92% to ↓14% [n.d.]	Stout et al. (2014) [Stout et al., 2014]
<i>P. trichocarpa</i>	<i>4CL</i>	antisense	yes	mountain, NC, USA	12	78 plants per line	2 years	1 year (2 y/o roots)	↓24% to WT [↓46% to ↓5%]	↓S/G to ↑S/G [↓S/G and ↑S/G]	n.d. [n.d.]	n.d. [n.d.]	↓92% to ↑24% [n.d.]	Stout et al. (2014) [Stout et al., 2014]
<i>P. tomentosa</i>	<i>4CL</i>	antisense	yes	Beijing, China	2	n.a.	5 years	5 years	↓8% to ↓6% [↓42% to ↓26%]	n.d. [n.d.]	WT [n.d.]	n.d. [n.d.]	WT [WT]	Wang et al. (2012) [Jia et al., 2004]
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>4CL</i>	fiber-specific suppression	yes	Shanghai, China	3	30 plants per line	1 year	1 year	↓~25% [↓21%]	n.d. [n.d.]	n.d. [n.d.]	n.d. [n.d.]	WT [WT]	Cao et al. (2020) [Cao et al., 2020]

Table 3. Biomass, lignin amount, and lignin composition of lignin-engineered trees grown in the field compared with greenhouse conditions.

(Continued on next page)

Species	Gene	Method	Tested in GH?	Location	# Lines	Field size	Field duration	Age of the stems at harvest	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	References
<i>P. deltooides</i> × <i>P. euramericana</i>	4CL	vessel-specific suppression	yes	Shanghai, China	3	30 plants per line	1 year	1 year	↓ ~20% [↓ 26%]	n.d. [n.d.]	n.d. [n.d.]	n.d. [n.d.]	↓ [WT]	Cao et al. (2020) [Cao et al., 2020]
<i>P. alba</i> × <i>P. glandulosa</i>	CSE1	CRISPR-Cas9	yes	Republic of Korea	1 or 3 ^a	n.a.	1 year	8 months or 1 year ^b	↓ ~24% [↓ up to 16%]	n.d. [n.d.]	↑ [n.d.]	n.d. [n.d.]	WT [WT]	Jang et al. (2021) [Jang et al., 2021]
<i>P. alba</i> × <i>P. glandulosa</i>	CSE2	CRISPR-Cas9	yes	Republic of Korea	1 or 3 ^a	n.a.	1 year	8 months or 1 year ^b	↓ 29% [↓ up to 16%]	n.d. [n.d.]	↑ [n.d.]	n.d. [n.d.]	WT [WT]	Jang et al. (2021) [Jang et al., 2021]
<i>P. tomentosa</i>	CCoAOMT	antisense	yes	Beijing, China	2	n.a.	5 years	5 years	↓ 10% to ↓ 9% [↓ 16%]	n.d. [n.d.]	↑ [n.d.]	n.d. [n.d.]	WT [WT]	Wang et al. (2012) [Jing et al., 2004]
<i>P. tremula</i> × <i>P. alba</i>	CCoAOMT	antisense	no	n.a.	1	n.a.	3 years	3 years	↓ 13%	↑ S/G	n.d.	↑	WT	Wei et al. (2008)
<i>P. tremula</i> × <i>P. alba</i>	CCR	sense and antisense	yes	Orléans, France	4	10 plants per line	8 years	2, 4, and 5 years ^c	↓ 47% to WT [↓ 30% to ↓ 22%]	↓ S/G, incorporation of FA [↓ S/G, incorporation of FA]	n.d. [n.d.]	↑ [n.d.]	↓ [WT]	Lepié et al. (2007) [Lepié et al., 2007]
<i>P. tremula</i> × <i>P. alba</i>	CCR	sense	yes	East Flanders, Belgium	2	120 plants per line	5 years	10 months	↓ 8% to ↓ 5% [↓ 30% to ↓ 22%]	WT S/G, incorporation of FA [↓ S/G, incorporation of FA]	↑ [↑]	n.d. [n.d.]	↓ [WT]	Van Acker et al. (2014) [Lepié et al., 2007]
<i>P. tremula</i> × <i>P. alba</i>	CCR	sense and antisense	yes	Orléans, France	2	120 plants per line	5 years	2 years	↓ 24% to ↓ 9% [↓ 30% to ↓ 22%]	↓ S/G to WT, incorporation of FA [↓ S/G, incorporation of FA]	↑ [↑]	n.d. [n.d.]	↓ [WT]	Van Acker et al. (2014) [Lepié et al., 2007]
<i>P. tremula</i> × <i>P. alba</i>	COMT	antisense	yes	Orléans, France	2	10 plants per line	5 years	2 years	WT [WT]	↓ S/G, incorporation of 5-OHG [↓ S/G, incorporation of 5-OHG]	n.d. [n.d.]	n.d. [↓]	WT [WT]	Pilate et al. (2002) [Lapierre et al., 1999; Van Doorselaere et al., 1995]
<i>P. tremula</i> × <i>P. alba</i>	COMT	antisense	yes	Orléans, France	2	10 plants per line	5 years	2 years (4 y/o roots)	WT [WT]	↓ S/G, incorporation of 5-OHG [↓ S/G, incorporation of 5-OHG]	n.d. [n.d.]	↓ [↓]	WT [WT]	Pilate et al. (2002) [Lapierre et al., 1999; Van

Table 3. Continued

(Continued on next page)

Species	Gene	Method	Tested in GH?	Location	# Lines	Field size	Field duration	Age of the stems at harvest	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	References
														Doorsselaere et al., 1995]
<i>P. tremula</i> × <i>P. alba</i>	COMT	antisense	yes	Berkshire, England	2	12 plants per line	4 years	4 years	WT [WT]	↓ S/G, incorporation of 5-OHG [↓ S/G, incorporation of 5-OHG]	n.d. [n.d.]	↓ [↓]	WT [WT]	Pilate et al. (2002) [Lapierre et al., 1999; Van Doorsselaere et al., 1995]
<i>P. tremula</i> × <i>P. alba</i>	COMT	antisense	yes	n.a.	2	n.a.	2 years	2 years	WT [WT]	↓ S/G, incorporation of 5-OHG [↓ S/G, incorporation of 5-OHG]	n.d. [n.d.]	↓ [↓]	WT [WT]	Lapierre et al. (1999) [Lapierre et al., 1999; Van Doorsselaere et al., 1995]
<i>P. tremula</i> × <i>P. alba</i>	CAD	sense and antisense	yes	n.a.	3	n.a.	2 years	2 years	↓ 4% to WT [↓ 4% to WT]	WT S/G, ↑ free phenolic G and S units [WT S/G]	n.d. [n.d.]	↑ [↑]	WT [WT]	Lapierre et al. (1999) [Lapierre et al., 1999]
<i>P. tremula</i> × <i>P. alba</i>	CAD	antisense	yes	Orléans, France	2	10 plants per line	5 years	2 years	Slightly ↓ [WT]	WT S/G, ↑ free phenolic G and S units [WT S/G]	n.d. [n.d.]	n.d. [↑]	WT [WT]	Pilate et al. (2002) [Baucher et al., 1996]
<i>P. tremula</i> × <i>P. alba</i>	CAD	antisense	yes	Orléans, France	2	10 plants per line	5 years	2 years (4 y/o roots)	Slightly ↓ [WT]	WT S/G, ↑ free phenolic G and S units [WT S/G]	n.d. [n.d.]	↑ [↑]	WT [WT]	Pilate et al. (2002) [Baucher et al., 1996]
<i>P. tremula</i> × <i>P. alba</i>	CAD	antisense	yes	Berkshire, England	2	12 plants per line	4 years	4 years	Slightly ↓ [WT]	WT S/G, ↑ free phenolic G and S units [WT S/G]	n.d. [n.d.]	↑ [↑]	WT [WT]	Pilate et al. (2002) [Baucher et al., 1996]
<i>Morus alba</i>	CAD	natural mutant ^d	no	Tsukuba, Japan	1	n.a.	n.a.	1 year	Lighter color in Mäule staining	n.d.	↑	↑	n.d.	Ikeda et al. (2021)

Table 3. Continued

(Continued on next page)

Species	Gene	Method	Tested in GH?	Location	# Lines	Field size	Field duration	Age of the stems at harvest	Effect on total lignin	Effect on lignin composition	Saccharification efficiency	Pulping efficiency	Biomass yield	References
<i>Morus alba</i>	CAD	natural mutant ^d	no	Tsukuba, Japan	1	n.a.	n.a.	n.a.	↓33%	↓S/G	n.d.	n.d.	n.d., lodging phenotype	Yamamoto et al. (2020)
<i>P. tremula</i> × <i>P. alba</i>	CAD	RNAi	yes	East Flanders, Belgium	3	240 plants per line	4 years	1 year	↓10% to ↓8% [↓10% to ↓6%]	↓S/G [↓S/G]	↑ [↑]	n.d. [n.d.]	↓ [WT]	De Meester et al. (2022b) [Van Acker et al., 2017]
<i>P. tremula</i> × <i>P. alba</i>	CAD	RNAi	yes	East Flanders, Belgium	3	240 plants per line	4 years	3 years (4 y/o roots)	WT [↓10% to ↓6%]	WT [↓S/G]	↑ [↑]	n.d. [n.d.]	↓ [WT]	De Meester et al. (2022b) [Van Acker et al., 2017]
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>LTF1</i>	fiber-specific suppression	yes	Shanghai, China	2	n.a.	1 year	1 year	↓ [↓43%]	n.d. [↓S/G]	↑ [n.d.]	n.d. [n.d.]	WT [WT]	Gui et al. (2020) [Gui et al., 2020]
<i>P. deltoides</i> × <i>P. euramericana</i>	<i>LTF1</i>	vessel-specific suppression	yes	Shanghai, China	2	n.a.	1 year	1 year	↓ [↓16%]	n.d. [↑S/G]	↓ [n.d.]	n.d. [n.d.]	↓ [↓]	Gui et al. (2020) [Gui et al., 2020]

Table 3. Continued

lines, number of lines with the intended transgenic construct or mutant allele (thus not including control lines); age of stem at harvest, if not explicitly stated otherwise, the age of the root is equal to that of the stem; n.d., not determined; n.a., not available; y/o, year-old; S/G, syringyl/guaiacyl ratio; S/V, syringaldehyde/vanillin ratio; FA, ferulic acid; 5-OHG, 5-hydroxyguaiacyl. Text in square brackets [] refers to data from greenhouse-grown trees of the same transgenic line, if available. For saccharification efficiency, see the corresponding reference for specific information about biomass pretreatments. Effect on total lignin is given as a percentage of the total lignin analyzed via different methods; see the corresponding reference for specific information.

^aOne line for lignin amount determination, three lines for biomass determination.

^bEight-month-old trees for total lignin determination, 12-month-old trees for biomass determination.

^cTwo-year-old trees for total lignin and lignin composition determination, 4-year-old trees for biomass yield determination, 5-year-old trees for chemical pulping experiments.

^dThe data presented are for the natural mutant Sekizaisou in comparison with other mulberry lines.

in the field (Lep   et al., 2007; Van Acker et al., 2017; Cao et al., 2020; De Meester et al., 2022a). Lignin reduction is also highly dependent on biotic and abiotic environmental factors determined by the field location. For example, the reduction in lignin in *4CL*-downregulated poplars grown for 2 years in the field was different depending on whether the lines were grown in mountain, coastal plain, or Piedmont regions of North Carolina (Stout et al., 2014). Similarly, a 2-year-old *CAD*-downregulated line exhibited different levels of lignin reduction depending on whether it was grown in a field in the UK or in a field in France (Pilate et al., 2002).

- (2) The lignin reduction and growth phenotype of trees engineered to produce less lignin are highly dependent on tree age. Indeed, consistent with the first conclusion, the lignin reduction in field-grown trees engineered to produce less lignin often becomes less pronounced throughout development and is generally smaller than in their greenhouse-grown clonal ramets. For example, *4CL*-downregulated lines grown in the greenhouse had severely reduced lignin amounts (up to 47% less lignin than the WT control), whereas the same transgenic lines grown on a mountain site field had mildly reduced lignin amounts (up to 8%) after 2 years and lignin amounts similar to that of the WT after 3 years of growth (Xiang et al., 2017). Similarly, greenhouse-grown *CAD*-downregulated poplars had 10% less lignin (Van Acker et al., 2017), whereas the same transgenic lines grown in the field displayed 10% less lignin after 1 year and had lignin amounts equal to that of the WT after 3 years of growth (De Meester et al., 2022a). The lignin content in 1-year-old greenhouse-grown *4CL*- and *CCoAOMT*-downregulated poplars was reduced by 16%–42%, whereas that in 5-year-old field-grown clonal ramets was reduced by only 6%–10% (Wang et al., 2012). This trend might be explained by the fact that greenhouse-grown trees continuously grow and develop new xylem because of the long-daylight regime typically used in greenhouses, but trees grown in the field in a temperate climate zone cease growth in late summer every year and may continue to lignify their cell walls—aided by the good neighbor cells—until they enter dormancy (Van Acker et al., 2014; De Meester et al., 2022a). Alternatively, these observations might be explained by the enhanced exposure to (multiple cycles of) environmental stresses in the field, such as drought, leading to enhanced lignin biosynthesis (Jubany-Mar   et al., 2009; Lee et al., 2012; Cesarino, 2019; Liu et al., 2021b). They might also be explained by fluctuations in the level of gene downregulation. Indeed, the extent of RNA interference (RNAi)-induced downregulation of a specific gene is dependent not only on the specific transfer DNA (T-DNA) integration event and thus the specific transgenic line but also on the individual plant and the specific environment. For example, substantial variation in the level of downregulation was observed among clonal ramets of the same *CCR2*-downregulated line, as judged from variation in the reddish-brown xylem phenotype associated with *CCR2* downregulation (Van Acker et al., 2014). To exclusively evaluate the effects of environment and age on lignin content reduction (and to exclude possible

interactions between the environment/tree age and the level of gene silencing), CRISPR-Cas9-generated lignin-engineered trees, whose gene knockdowns or knockouts are stable by definition, will need to be tested in different locations over multiple years (Zhou et al., 2015; De Meester et al., 2020; de Vries et al., 2021a).

In many cases, the enhanced wood-processing efficiencies typically found for greenhouse-grown lignin-engineered trees are also observed for field-grown trees, albeit sometimes to a lesser extent and/or accompanied by a yield penalty (Tables 1 and 3). Although there may be ways to circumvent the frequently observed yield penalty in lignin-engineered trees (see below), the observation that the lignin content of trees RNAi-engineered to produce less lignin often gradually increases with age, generally implying that the processing efficiency gradually decreases, may call for a significant revision of research strategies. If similar observations are made for CRISPR-Cas9-generated lignin-engineered trees, a better strategy to deploy lignin-engineered trees for applications might be to grow the trees in very short-rotation coppice (e.g., harvesting every year or 2 years). Alternatively, engineering the lignin composition instead of the lignin amount or reducing the relative amounts of lignin in the cell wall by increasing cellulose or matrix polysaccharides (e.g., by making use of transcription factors) are valuable strategies.

ORIGINS OF THE YIELD PENALTY OFTEN OBSERVED IN LIGNIN-ENGINEERED TREES

Lignin-modified plants that show the greatest improvement in processing efficiency often also exhibit biomass yield penalties (also termed lignin modification-induced dwarfism [LMID]), thereby limiting the benefits of their enhanced processing efficiency (Chen and Dixon, 2007; Bonawitz and Chapple, 2013; Van Acker et al., 2014; Muro-Villanueva et al., 2019). Because lignin provides rigidity and hydrophobicity to plant cell walls (Turner and Somerville, 1997), one might expect this growth reduction to be directly correlated with the level of lignin reduction. However, the literature shows that some transgenic poplars with severely reduced amounts of lignin had biomass yields similar to the WT, whereas poplars with unaltered or mildly reduced lignin amounts displayed severe yield penalties. For example, *4CL*-downregulated poplars had up to 40%–45% less lignin and normal or increased growth (Hu et al., 1999; Li et al., 2003; Jia et al., 2004), whereas *C4H*-downregulated poplars had 13% less lignin and reduced growth (Kim et al., 2020). Similarly, *PAL*-downregulated lines (i.e., lines i7-10 and i7-2) had severely reduced amounts of lignin (–57% to –42%) and mildly reduced heights (–17% to –7%), whereas *HCT*-downregulated trees (i.e., line i19-4) had moderately reduced lignin amounts (–21% to –17%) and severely reduced heights (–65% to –43%) (Wang et al., 2018). Wang et al. (2018) also showed that the growth of lignin-engineered trees is not associated with a particular lignin subunit composition or specific linkages. If not changes in lignin content and composition, what mechanisms give rise to the impaired growth observed in many lignin-engineered trees (and other plants)? Several hypotheses explaining LMID in lignin-engineered plants have been postulated (Bonawitz and Chapple, 2013; Muro-Villanueva et al., 2019; Ha et al., 2021). Below, we

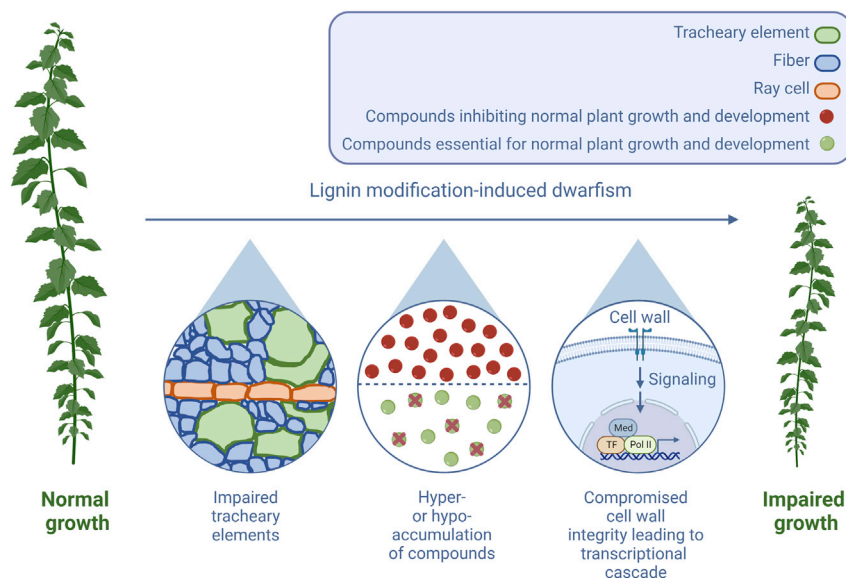


Figure 3. Hypotheses explaining lignin modification-induced dwarfism (LMID) in forest trees.

First, LMID might be caused by impaired functioning of TEs, resulting in perturbed water and nutrient transport in the stem. Second, a block in the phenylpropanoid pathway could lead to the accumulation of growth-inhibitory compounds or prevent the biosynthesis of compounds essential for growth. Third, alterations in the cell wall might be detected by cell-wall integrity sensors that activate a transcriptional response requiring Mediator (Med). TF, transcription factor; Pol II, RNA polymerase II.

Second, LMID might be caused by hyper- or hypoaccumulation of pathway intermediates and/or derivatives. When a certain enzymatic conversion in the lignin biosynthesis pathway is perturbed, compounds located upstream of this conversion or their derivatives may accumulate, and the abundance

of compounds located downstream of this conversion will decrease. In addition, a transcriptional cascade aimed at increasing flux through the pathway is activated to compensate for the loss of lignin building blocks (e.g., by the activation of shikimate and phenylpropanoid pathways), resulting in the (additional) toxic buildup of compounds because the pathway remains blocked (Vanholme et al., 2012). Hyper- or hypoaccumulation of phenylpropanoid-related compounds can have a strong effect on the stress- or defense-related transcriptome (Tsai et al., 2020) and, hence, on the abundance of other growth-related compounds, potentially leading or at least contributing to the observed yield penalties. For example, the *Arabidopsis ref3* mutant accumulates high levels of cinnamate-derived compounds owing to its reduced C4H activity. Cinnamate hyperaccumulation in *ref3* mutants impairs auxin signaling, which might underlie the observed yield penalty (Schilmüller et al., 2009; Steenackers et al., 2019; El Houari et al., 2021). *Arabidopsis* and *Medicago* HCT-deficient plants accumulate high levels of the stress hormone salicylic acid (SA), which negatively influences stem height in the transgenic plants (Gallego-Giraldo et al., 2011a). Reducing SA levels in these plants restored plant growth. Furthermore, overexpression of *LAC4* or *LAC17* in high-monolignol glucoside-containing lines (*MYB58-OE* or *MYB63-OE*) decreases levels of monolignol glucosides and rescues impaired plant growth (Perkins et al., 2020). The growth-inhibitory effect of specific hyperaccumulating phenylpropanoids and phenylpropanoid-derived compounds might explain why, e.g., *HCT*-downregulated poplars show severe growth defects, whereas *PAL*-downregulated lines show no/very mild growth defects (Wang et al., 2018). Based on current insights into the phenylpropanoid pathway (Figure 1), *HCT*-downregulated lines are expected to accumulate the HCT substrate *p*-coumaroyl-CoA and its derivatives that remain soluble and potentially interfere with cellular processes, whereas *PAL*-downregulated lines accumulate the substrate phenylalanine, which can be further metabolized in primary processes such as protein synthesis. Following this rationale, valuable lignin-engineering strategies might focus on targeting enzymes that work upstream of C4H, resulting in a buildup of substrates that could

summarize these hypotheses (which were based mainly upon observations in *Arabidopsis*) and discuss whether experimental support for each model also exists in trees (Figure 3).

First, LMID in lignin-modified trees could be caused by impaired functioning of the tracheary elements (TEs; vessels in hardwood, tracheids in softwood) as a result of (i) vascular collapse (Lepié et al., 2007; Coleman et al., 2008; Wagner et al., 2009; Zhou et al., 2018; De Meester et al., 2020; de Vries et al., 2021a), (ii) blockage of TEs with tyloses and phenolic deposits (Kitin et al., 2010), or (iii) enlarged vessels (which generally enhance xylem vulnerability to embolism) combined with reductions in relative water content and free water available to support the hydraulic network (De Meester et al., 2022b). Consequently, the stem sap flow and/or hydraulic conductance may be perturbed, potentially leading to enhanced susceptibility to drought stress and/or impaired tree growth (Cochard et al., 2004; Coleman et al., 2008; Cao et al., 2020; White, 2021; De Meester et al., 2022b). Impaired TEs have been observed in growth-impaired angiosperm trees with reduced expression of *HCT* (Zhou et al., 2018), *C3'H* (Coleman et al., 2008; Zhou et al., 2018), *4CL* (Kitin et al., 2010; Voelker et al., 2010, 2011), *CSE* (de Vries et al., 2021a), *CCR* (Lepié et al., 2007; De Meester et al., 2020), and *CAD* (De Meester et al., 2022b), as well as growth-impaired gymnosperm trees with reduced expression of *4CL* (Wagner et al., 2009). Similar to findings in *Arabidopsis* (Turner and Somerville, 1997; Brown et al., 2007; Peña et al., 2007), collapsed TEs were also found in (growth-impaired) cellulose- and hemicellulose-deficient trees, highlighting that not only lignin but also other cell-wall components are important for vascular integrity in trees (Joshi et al., 2011; Lee et al., 2011; Yu et al., 2013). As in *Arabidopsis* (Yang et al., 2013; Vargas et al., 2016; De Meester et al., 2018), the impaired TE hypothesis is supported by the observation that LMID in trees can be (largely) avoided by targeting the lignin reduction specifically to fibers while still allowing sufficient lignin to be deposited in TEs (see also below) (Cao et al., 2020; Gui et al., 2020; De Meester et al., 2021).

of compounds located downstream of this conversion will decrease. In addition, a transcriptional cascade aimed at increasing flux through the pathway is activated to compensate for the loss of lignin building blocks (e.g., by the activation of shikimate and phenylpropanoid pathways), resulting in the (additional) toxic buildup of compounds because the pathway remains blocked (Vanholme et al., 2012). Hyper- or hypoaccumulation of phenylpropanoid-related compounds can have a strong effect on the stress- or defense-related transcriptome (Tsai et al., 2020) and, hence, on the abundance of other growth-related compounds, potentially leading or at least contributing to the observed yield penalties. For example, the *Arabidopsis ref3* mutant accumulates high levels of cinnamate-derived compounds owing to its reduced C4H activity. Cinnamate hyperaccumulation in *ref3* mutants impairs auxin signaling, which might underlie the observed yield penalty (Schilmüller et al., 2009; Steenackers et al., 2019; El Houari et al., 2021). *Arabidopsis* and *Medicago* HCT-deficient plants accumulate high levels of the stress hormone salicylic acid (SA), which negatively influences stem height in the transgenic plants (Gallego-Giraldo et al., 2011a). Reducing SA levels in these plants restored plant growth. Furthermore, overexpression of *LAC4* or *LAC17* in high-monolignol glucoside-containing lines (*MYB58-OE* or *MYB63-OE*) decreases levels of monolignol glucosides and rescues impaired plant growth (Perkins et al., 2020). The growth-inhibitory effect of specific hyperaccumulating phenylpropanoids and phenylpropanoid-derived compounds might explain why, e.g., *HCT*-downregulated poplars show severe growth defects, whereas *PAL*-downregulated lines show no/very mild growth defects (Wang et al., 2018). Based on current insights into the phenylpropanoid pathway (Figure 1), *HCT*-downregulated lines are expected to accumulate the HCT substrate *p*-coumaroyl-CoA and its derivatives that remain soluble and potentially interfere with cellular processes, whereas *PAL*-downregulated lines accumulate the substrate phenylalanine, which can be further metabolized in primary processes such as protein synthesis. Following this rationale, valuable lignin-engineering strategies might focus on targeting enzymes that work upstream of C4H, resulting in a buildup of substrates that could

be used in primary metabolism (thus avoiding the accumulation of useless and potentially toxic carbon sinks). For example, the transcription factors *PtrHSFB3-1* and *PtrMYB092* have been shown to activate transcription of multiple genes encoding enzymes in the phenylpropanoid and monolignol-specific pathways, including PAL family enzymes and most likely also enzymes upstream of PAL (Liu et al., 2021a). Poplar knockout mutants in either *PtrHSFB3-1* or *PtrMYB092* display a low lignin content (up to –17% or –27%, respectively) without adverse effects on biomass yield (Liu et al., 2021a). Notably, although several lignin-engineered trees have been shown to hyper- or hypoaccumulate a plethora of compounds (e.g., Lep   et al., 2007; Saleme et al., 2017; De Meester et al., 2020; de Vries et al., 2021a), to date, no support for a link between the abundance of a specific compound and growth has been found in trees. This is due at least in part to the fact that the metabolic response to pathway perturbations is complex, involving many unknown metabolites.

Third, LMID could be triggered by a cell-wall integrity system that senses defects in the cell wall and consequently activates a cascade of transcriptional changes to overcome such defects. This reallocation of resources toward the constitutive production of cell-wall components and/or defense compounds might come at the expense of plant growth. Indeed, judging from their increased expression of pathogenesis-related genes, *HCT*-deficient alfalfa and *Arabidopsis* plants exhibit constitutive activation of defense responses (Gallego-Giraldo et al., 2011a, 2011b). More recently, a study with *HCT*- and *CCR1*-deficient *Arabidopsis* showed that defense gene induction and release of elicitors result from the ectopic expression of *ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1* (*ADPG1*), which encodes a pectin-degrading enzyme (Gallego-Giraldo et al., 2020). The role of transcriptional changes in LMID has been demonstrated by the (partial) restoration of biomass yield upon mutation of *Med5A/5B* subunits of the transcriptional coregulator Mediator in *Arabidopsis c3'h1* mutants (Bonawitz et al., 2014). Nevertheless, the role of Mediator in LMID of lignin-engineered trees has not been studied to date.

It should be noted that the occurrence, cause, and severity of LMID depend on the position of the perturbation in the pathway (as described above for *PAL*- versus *HCT*-downregulated poplars). For example, although the growth of dwarfed *HCT*-deficient *Arabidopsis* is restored by reducing SA levels (Gallego-Giraldo et al., 2011a), other phenylpropanoid mutants exhibit dwarfed phenotypes and decreased SA levels (Huang et al., 2010) or grow normally and have increased SA levels (Bonawitz et al., 2014). Although the growth of *c3'h1* *Arabidopsis* mutants is largely restored by mutating the *med5a/5b* subunits of the Mediator complex (Bonawitz et al., 2014), the stunted growth of *c4h* and high-monolignol *MYB63*-OE *Arabidopsis* mutants is not recovered by disrupting *Med5A/5B* (Bonawitz et al., 2014; Perkins et al., 2020). Finally, the species, the specific genotype, and the environment play roles in the expression of the phenotype, as illustrated by *4CL*-downregulated poplar; the growth of *4CL*-deficient *Populus tomentosa* in a field in China was increased by 8% (Tian et al., 2013), whereas *4CL*-deficient *P. tremula* × *P. alba* grown in a field in Oregon displayed yield penalties. These differences may be due to different genetic backgrounds, different levels of *4CL* downregulation, or different environments.

OVERCOMING LMID IN LIGNIN-ENGINEERED TREES

Despite the fact that our understanding of the potential reasons for LMID remains fragmentary, strategies can be designed to eliminate or at least mitigate LMID by, e.g., avoiding impaired TEs, avoiding the (excessive) buildup or shortage of certain compounds, or avoiding the (excessive) triggering of the cell-wall integrity-monitoring pathway. Moreover, these potential causes might be interconnected; relieving vascular collapse might also relieve the triggering of the cell-wall-monitoring pathway and the buildup of (toxic) compounds and vice versa.

To avoid LMID, the level of target gene downregulation is of major importance. For example, *CSE*-downregulated *P. tremula* × *P. alba* trees generated by RNAi had 25% less lignin and normal growth (Saleme et al., 2017), whereas CRISPR-Cas9-generated *CSE*-knockout *P. tremula* × *P. alba* had 35% less lignin and perturbed growth (de Vries et al., 2021a). In addition to RNAi, gene editing or screening of natural populations can also be used to obtain a range of lines with various levels of target enzyme activity. For example, *CCR2*-knockout *P. tremula* × *P. alba* generated using CRISPR-Cas9 were extremely dwarfed and could barely survive when grown in soil (De Meester et al., 2020). By contrast, *P. tremula* × *P. alba* harboring one knockout *CCR2* allele and one weak *CCR2* allele (with a 3-bp deletion, resulting in a 114I115A-to-114T conversion in the *CCR2* protein), had 10% less lignin, grew normally, and showed improved wood-processing efficiency (De Meester et al., 2020). In a second example, *HCT*-downregulated poplars made with RNAi had severe growth reductions (Wang et al., 2018), whereas a naturally occurring poplar *HCT* mutant (homozygous for a point mutation leading to a truncated HCT protein lacking 73 amino acids at the C-terminal end) had a significantly altered lignin composition but showed normal growth (Vanholme et al., 2013a).

Next to residual enzyme activity, the spatial distribution of the (remaining active) lignin biosynthesis enzyme also seems to be critical for avoiding LMID. In *Arabidopsis*, reintroduction of lignin biosynthesis specifically in the TEs (and not the fibers) of *c4h*, *cse*, and *ccr1* mutants (largely) restores the collapsed TEs and stunted growth (Yang et al., 2013; Vargas et al., 2016; De Meester et al., 2018). However, because of the presence of “good neighbors for lignification” monolignols synthesized in TE cells not only lignify the TE cell wall but also contribute to cell-wall lignification of neighboring cells (Pesquet et al., 2013; Smith et al., 2013, 2017; De Meester et al., 2018). For example, in *Arabidopsis ccr1* mutants expressing *CCR1* under the control of a vessel-specific promoter (*ccr1 ProSNBE:AtCCR1*), the production of monolignols is restricted to TE cells, but xylary fibers (and not interfascicular fibers) also show enhanced lignin deposition. In poplar, expression of *ProSNBE:AtCCR1* in CRISPR-Cas9-generated *ccr2* mutants results in TE- and ray-specific monolignol biosynthesis and restores vascular integrity and biomass yield (De Meester et al., 2021). In transgenic lines in which monolignols are abundantly produced by TEs and ray cells, these monolignols migrate across multiple cell layers into adjoining and non-adjoining fibers, resulting in a restoration of lignin amount in all xylem cells. In lines whose supply of monolignols is limited, monolignols are incorporated only into the cell walls of the TEs and rays producing them and their adjoining cells,

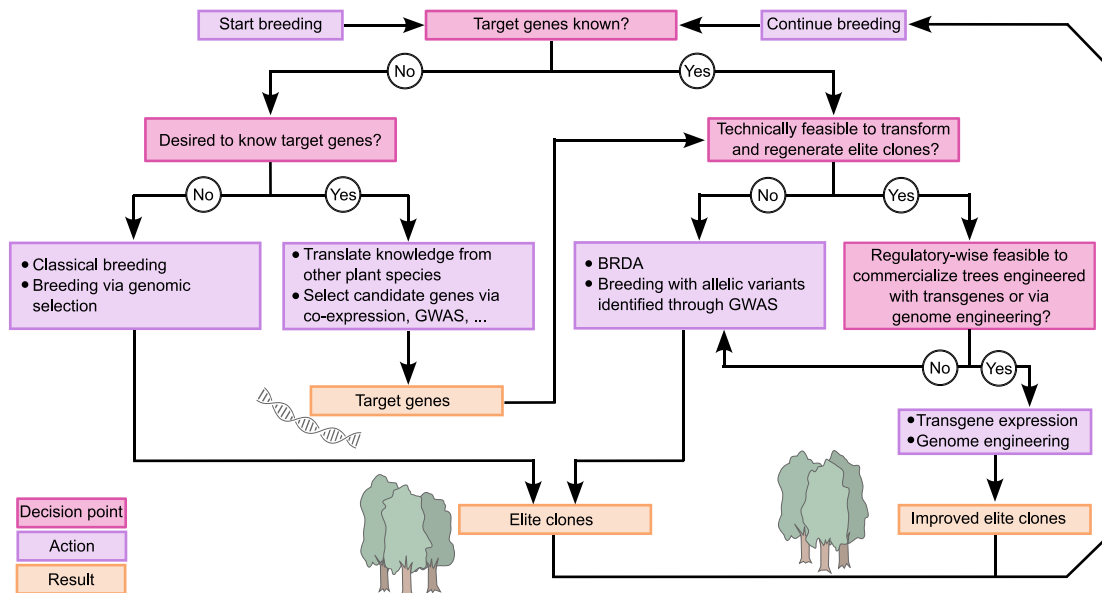


Figure 4. Simplified flowchart reflecting the integration of various breeding strategies for lignin engineering in forest trees.

In general, the most efficient breeding strategy uses a combination of techniques as follows: first, breed via classical breeding and genomic selection for a series of traits such as wood quality, disease resistance, and growth architecture, thus producing elite clones. Next, use transgene or genome engineering to further improve or introduce the desired lignin traits, thereby avoiding the loss of genetic constitution in elite lines. BRDA, breeding with rare defective alleles; GWAS, genome-wide association studies.

resulting in the desired fiber hypolignification. Alternatively, fiber hypolignification can be achieved by repressing lignin biosynthesis in fiber cells of poplar by, e.g., fiber-specific downregulation of *4CL1* or fiber-specific expression of the constitutive repressor *LTF1^{AA}* (Cao et al., 2020; Gui et al., 2020). In both cases, the transgenic poplars displayed hypolignified fibers but normal TEs and growth. In the *4CL1*-engineered poplars, monolignol-producing TEs act as good neighbors for lignification of adjoining fibers but not fibers located further away (Cao et al., 2020). However, no (or very little) lignification of both adjoining and non-adjoining fibers was observed in *LTF1^{AA}* poplars, probably because all genes involved in lignin biosynthesis in the fibers were suppressed, including those encoding cell-wall-localized laccases and peroxidases involved in dehydrogenation of the lignin monomers (Gui et al., 2020). By contrast, when lignin biosynthesis was specifically allowed in fiber cells, leaving the TEs hypolignified, the transgenic poplars had collapsed TEs and severe growth perturbations (Cao et al., 2020; Gui et al., 2020). These observations show that the presence of sufficient lignin in the TEs is crucial for avoiding LMID in trees, whereas hypolignification of the fibers does not appear to affect growth.

BREEDING LIGNIN TRAITS IN FOREST TREES

Unlike fruit trees that have been domesticated for thousands of years, forest trees were not actively subjected to domestication until the 1950s (Lebedev et al., 2020). Nevertheless, the use of traditional breeding to improve lignin traits in forest trees has great potential. Large variations in lignin amount (15.7%–27.9% and 24.4%–32.1%) and S/G ratio (1.0–3.0 and 1.8–4.2) are present in undomesticated *Populus trichocarpa* (Studer et al.,

2011) and *Eucalyptus grandis* × *Eucalyptus urophylla* F2 full-sib families (Marco de Lima et al., 2019), respectively. Thus, substantial improvements in lignin content and composition of forest trees are possible by traditional breeding alone (Studer et al., 2011). As traditional breeding is time consuming owing to long generation times and the fact that wood quality traits are typically best evaluated at rotation age (Ahmar et al., 2021), tree breeding can benefit from the use of genomic information (Figure 4). For instance, genomic selection has substantially accelerated the speed of forest tree breeding (Grattapaglia et al., 2018; Howe et al., 2020; Lebedev et al., 2020; Ahmar et al., 2021; Grattapaglia, 2022). This technique enables the prediction of multiple traits based on thousands of molecular markers and therefore depends on low-cost, high-throughput sequencing techniques (Grattapaglia and Resende, 2011; Rambolarimanana et al., 2018; Grattapaglia, 2022). The underlying genes and alleles that cause the desired trait—let alone the biochemical mechanism behind it—usually remain unknown. Genomic selection is currently used to optimize lignin traits in eucalyptus (Rambolarimanana et al., 2018; Marco de Lima et al., 2019; Bouvet et al., 2020).

Complementary approaches to improve lignin properties in trees are breeding techniques that target specific genes known to be involved in lignin biosynthesis (Figure 4). As described above, many genes involved in lignification are already known (Figure 1). These have been discovered over the past decades through fundamental research on both model organisms and tree species, e.g., by forward genetic approaches and after the selection of candidate genes based on co-expression analysis followed by reverse genetic approaches. Genome-wide association studies (GWAS) are of particular interest because GWAS can be used to identify genes/alleles that are causal for a specific trait in a population (Fahrenkrog et al.,

2017). For instance, association genetics in different poplar species has revealed associations between lignin amount or composition and genes encoding β -TUBULIN 15, CCoAOMT1, an FAD-binding domain-containing protein, ADP-ribosylation factor (ARF) GTPase-activating protein (GAP) domain 11, the KANADI transcription factor, and EPSPS (Guerra et al., 2013; Porth et al., 2013; Muchero et al., 2015; Xie et al., 2018, 2020; Yao et al., 2021). The roles of CCoAOMT1 and EPSPS in lignification have been validated by reverse genetics (Meyermans et al., 2000; Zhong et al., 2000; Xie et al., 2020). Further reverse genetics support is needed to validate the roles of β -TUBULIN 15, the FAD-binding domain-containing protein, ARF-GAP domain 11, and KANADI in lignin biosynthesis.

If the causal genes for a given trait are known, traditional tree breeding can also be accelerated by screening large populations of trees by next-generation sequencing for individuals with rare alleles of target genes and by introducing these genes into breeding programs (Marroni et al., 2011; Vanholme et al., 2013a), a strategy termed breeding with rare defective alleles (BRDA). The identification of a natural mutant allele of *HCT1* that increased the frequency of H units in *Populus nigra* served as a proof of concept for the use of this strategy to alter lignin traits in poplar (Vanholme et al., 2013a). The potential of using natural allelic variation was also illustrated by the discovery of a natural *cad* null mutant in *Pinus taeda* L. (loblolly pine) (MacKay et al., 1997). Lignin was lowered by about 10% and was composed of 15% coniferaldehyde and 30% dihydroconiferyl alcohol in the mutant, whereas these values were 7% and 3%, respectively, in control pine (MacKay et al., 1997; Ralph et al., 1997). In addition, the *Morus alba* (mulberry) cultivar Sekizaisou was revealed to be a natural *cad* null mutant (Yamamoto et al., 2020). The tree showed reduced lignin levels with increased incorporation of hydroxycinnamaldehydes, improved wood delignification efficiency, and increased pulp yield under alkaline pulping conditions compared with a set of reference genotypes that lacked the *cad* mutation (Ikeda et al., 2021). Use of such naturally occurring rare defective alleles in breeding programs can be useful if they are present in the breeding germplasm, but modern breeding techniques enable engineering of the desired alleles directly into elite genotypes.

Modern breeding techniques, such as genetic engineering via transgene expression and gene editing, have opened up new possibilities in forest tree breeding (Chanoca et al., 2019; Min et al., 2022). Once elite genotypes have been generated by classical breeding, use of these modern breeding techniques enables them to be further improved without breaking up their genetic constitution. Moreover, these techniques enable the introduction of traits that are not present in the species and the generation of allelic variants that are absent or rare in the population (Goralogia et al., 2021). Use of transgenes enables alteration of target gene expression levels and introduction of genes from pathways that are not found in natural populations. Lignin modifications that rely on the biosynthesis of alternative monomers, such as coniferyl ferulate and sinapyl-*p*-coumarate, can therefore be accomplished via transgenic approaches (Wilkerson et al., 2014; Lapierre et al., 2021). Although small-scale field trials have shown the benefits of lignin engineering (Table 3) (Lapierre et al., 1999; Pilate et al., 2002; Wei et al., 2008; Wang et al., 2012; Tian et al., 2013; Cao et al., 2020; Gui

et al., 2020), to our knowledge, no transgenic elite lines with altered lignin are currently grown in commercial forestry applications because of the expensive regulatory burden that was installed for genetically modified plants decades ago and is no longer consistent with current scientific data (Strauss et al., 2015, 2019; Custers et al., 2016).

Genetically improved trees obtained via gene editing have also not yet made it into commercial plantations. Nevertheless, the potential of gene editing to engineer cell wall traits in forest trees is great. From a regulatory standpoint, gene-editing techniques have the advantage over genetic engineering, as there is relatively swift regulation in many regions of the world, including major forestry countries such as Canada and Brazil, for bringing gene-edited trees to the market (Ellens et al., 2019; Nepomuceno et al., 2020; Jenkins et al., 2021). However, at least for now, in the European Union (EU), organisms obtained by gene editing are subject to the same regulatory obligations as those obtained by genetic engineering (Court of Justice of the European Union, 2018). Also, from a technological perspective, gene editing has a number of advantages. Gene editing enables, by definition, more stable engineering than RNAi-based gene downregulation (Van Acker et al., 2013; De Meester et al., 2022a). In addition, individual genes from a gene family can be altered without affecting close gene family members. For instance, when *4CL1* was mutated via CRISPR-Cas9 in poplar, *4CL5* (which shows 89% nucleotide identity with *4CL1*) remained unaltered (Zhou et al., 2015), and *CSE1* and *CSE2* have been independently mutated via CRISPR-Cas9 in poplar despite their 88% nucleotide identity (de Vries et al., 2021a; Jang et al., 2021). Gene editing also enables the stable engineering of weak alleles. For instance, a poplar line with one *ccr2* knockout allele and one *ccr2* weak allele has a stably reduced lignin content and improved wood processing efficiency (De Meester et al., 2021). Furthermore, genome editing allows multiple genes to be edited simultaneously through multiplex editing; e.g., to edit multiple genes of the same pathway or to stack lignin traits with yield and disease-resistance traits.

However, an important limitation of the use of CRISPR-Cas in trees is that the T-DNA with the CRISPR-Cas sequence, which is currently stably integrated into the genome, must be eliminated before unconfined field release in order to take advantage of the low regulatory burden and swift regulation of gene-edited crops (Dalla Costa et al., 2020). Elimination of the T-DNA via crossing and Mendelian segregation is not preferred because it is time consuming and would break up the genetic constitution of highly heterozygous commercially relevant elite lines, leading to a loss of desired traits (Norelli et al., 2017). More research and development are needed to obtain efficient protocols for generating transgene-free gene-edited trees, as has already been successfully achieved for many other plant species (reviewed in He et al., 2022).

CONCLUDING REMARKS AND PERSPECTIVES

Modification of lignin to improve wood processing has been successfully achieved in several forest tree species. However,

commercialization of trees with reduced lignin levels or modified lignin is hindered by a number of factors. First, lignin engineering often results in lower biomass yields. Future efforts should therefore be made to address this issue, e.g., by tuning enzyme activity to obtain more subtle shifts in lignin levels, by reducing lignin content only in fibers, and by mutating genes located upstream of C4H (thus avoiding the accumulation of phenylpropanoids to potentially toxic levels) or genes encoding transcription factors that affect the entire lignin biosynthetic pathway. A second limitation is the lack of data on field-grown lignin-modified trees. Field trials of the most promising lignin-engineered lines are essential for the translation of basic research into applications. Importantly, in multi-year field trials, gradual fading of the reduction in wood lignin content of trees RNAi-engineered to produce less lignin has often been observed. Field trials of CRISPR-Cas-engineered trees are needed to further investigate whether this is a general phenomenon or whether confounding factors, such as variations in the level of downregulation caused by RNAi, are at play. A third hurdle is the lack of data on lignin engineering in elite lines. Here, establishing transformation protocols for elite lines would be helpful. In addition, protocols for gene editing that avoid the integration of the CRISPR-Cas-encoding DNA would further facilitate field studies and commercialization of new varieties. Furthermore, lignin engineering would benefit from evaluating the effects of stacking different lignin traits and stacking lignin traits with other desirable forestry traits (e.g., yield, wood density, pest resistance). Therefore, multiplex gene-editing strategies in which edits in different genes are stacked in a single elite line, followed by high-throughput screening of the engineered lines for combinations of edits, would further accelerate the development of lignin-engineered commercial forest trees.

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