Review

Effects of altered lignin biosynthesis on phenylpropanoid metabolism and plant stress

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Modification of lignin in bioenergy feedstocks has become a common strategy to increase saccharification and biofuel yield. The lignin biosynthetic pathway in several plant species has been dissected and key enzymes have been manipulated in transgenic plants. Recent analyses of lignin-modified plants have shown that decreasing lignin biosynthesis can alter carbon flow within the phenylpropanoid pathway and indirectly affect the synthesis of other secondary metabolites, many of which can play important roles in plant–environment interactions. In addition, lignin modifications have also been shown to induce the expression of various stress response-related genes. Examining and understanding these indirect effects of lignin modification on stress-related processes are essential, since they could ultimately impact the performance of low-lignin bioenergy feedstocks under agronomic field conditions. Recent efforts to characterize such effects will be discussed in this review.

While lignocellulosic feedstocks represent a promising renewable and sustainable alternative to petroleum-based fuels, high production costs associated with conversion processes currently prevent them from being economically viable for large-scale implementation [1]. The production of biofuels from lignocellulosic feedstocks requires the depolymerization of cell wall carbohydrates into simple sugars that can be utilized during fermentation. However, the desired cellulose microfibrils are surrounded by a matrix of lignin and hemicellulose, which greatly inhibits their accessibility to hydrolytic enzymes [1,2]. Lignin is a phenolic polymer that reinforces the secondary cell wall, confers structural integrity to the plant, aids in water transport and also plays an important role in plant responses to various environmental stresses. The presence of lignin in plant cells has been identified as a major contributor to the resistance of converting cell walls into fuel precursors [3]. Expensive and energy-intensive thermochemical pretreatments are generally required to disrupt the lignin–polysaccharide barrier and allow better access of the cellulose to hydrolysis prior to fermentation. An alternative approach for improving the accessibility of cell wall sugars and reducing the need for pretreatment is through genetic engineering of the lignin biosynthetic pathway. Reducing lignin content or modifying its composition can be achieved by downregulating or overexpressing genes involved in either lignin biosynthesis or its regulation [3–5].

Early studies from a decade or more ago that contributed to our current understanding of lignin biosynthesis and its manipulation focused on poplar (*Populus* spp.) to improve pulping performance [6,7], as well as forage species, such as alfalfa (*Medicago sativa* L.) and tall fescue (*Festuca arundinacea*), for improving digestibility [8–10]. A great deal of insight into lignin engineering has also been achieved through studies with the model species *Arabidopsis thaliana* [11] and tobacco (*Nicotiana tabacum*) [12]. Because the lignin biosynthetic pathway appears to be highly conserved among plant species, many of the genetic engineering strategies that have proven to be successful in model species can also be applied to lignocellulosic feedstocks [4]. Of particular importance to biofuel-related studies, *A. thaliana* has been identified as

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Key terms

Recalcitrance: The resistance of lignocellulosic biomass to enzymatic breakdown into fermentable sugars.

Phenylpropanoid pathway: Metabolic pathway responsible for the synthesis of diverse classes of plant secondary metabolites that play roles in developmental and stress-related processes.

Transcription factors: Proteins that regulate gene expression by binding to specific genes and promoting or repressing their transcription. The over-expression of transcription factors that downregulate the expression of lignin biosynthetic genes is an effective method for reducing lignin content.

a relevant model system for studying the effects of lignin modification on cell wall **recalcitrance**. Cell wall phenotypes in *Arabidopsis* have been translatable to commercial dicot species such as *M. sativa* and *Populus* spp. [13]. Currently, considerable research has focused on developing strategies for the genetic improvement of bioenergy feedstock candidate species including switchgrass (*Panicum virgatum* L.), sorghum (*Sorghum bicolor*), willow (*Salix* spp.), *Miscanthus*, sugarcane (*Saccharum* spp.) and poplar [14]. Transgenic modifications are likely to be the most direct way of improving the quality of lignocellulosic biomass for cost-effective conversion into biofuels [15], and manipulation of the lignin biosynthetic pathway has thus far proven to be an effective route for increasing saccharification efficiency and/or ethanol yield in greenhouse-grown switchgrass, field-grown poplar, and greenhouse- and field-grown sugarcane [16–23]. Additionally, opportunities for genetic engineering of lignin in other potential biofuel species might also exist. Sorghum mutants with low lignin content from brown mid-rib mutations are known for having enhanced digestibility and saccharification [24,25]. The lignin biosynthetic genes underlying some of these mutations have been identified, and may represent potential targets for downregulation [26–28].

The majority of studies with lignin-modified plants have primarily focused on the effects of lignin pathway modifications on lignin content and composition, and the associated changes in pulping performance, digestibility or recalcitrance to saccharification. More in-depth characterization of lignin-modified plants at the transcript, protein and metabolite level have revealed that these modifications can have unexpected effects on other metabolic processes beyond lignin biosynthesis. Monolignols, the lignin monomers, are synthesized through the lignin-specific branch of the phenylpropanoid metabolic pathway. Phenylpropanoid metabolism encompasses a network of metabolic pathways responsible for synthesizing a wide variety of secondary metabolites that play various roles in developmental and stress-related processes **(Figure 1)** [29,30], and several studies show that suppressing lignin biosynthesis can alter metabolic channeling within the **phenylpropanoid pathway** and differentially affect the synthesis of other secondary metabolites [31–34].

In addition to altering phenylpropanoid metabolism, modifications to lignin biosynthesis can also be associated with changes in how plants respond to biotic and abiotic stresses. The accumulation of lignin in response to pathogen infection, wounding and mechanical damage is an important defense mechanism in plants [35], and it has long been presumed that a reduction in lignin content would render plants more susceptible to such stresses. However, recent studies suggest that changes in stress susceptibility in lignin-modified plants are not necessarily direct results of reducing lignin content, but can be influenced by indirect effects of lignin pathway perturbations on stress-related metabolism and gene expression. Some reports with low-lignin mutant and transgenic plants indicate that the accumulation or suppression of certain secondary metabolites resulting from the alterations to phenylpropanoid metabolism can affect how plants interact with stresses [34,36–38]. Additionally, recent studies have revealed an increase in the expression of stress-related genes in lignin-modified plants, which some reports hypothesize to be triggered by the altered structural organization of the cell wall [31,39,40]. Evaluation of these plants under stress in controlled greenhouse or laboratory environments have indicated that these modifications to secondary metabolism and/or stress-related gene expression can interfere with or enhance the ability of the plant to tolerate stresses.

Because of the influence of lignin modification on plant stress, it is possible that altering lignin biosynthesis in second-generation bioenergy feedstocks could impact their performance in the field. In order to be commercially competitive, bioenergy crops should possess a number of desirable agronomic traits, including high biomass productivity under minimal agronomic inputs, high water and nutrient use efficiency, a relative resistance to pathogen and insect pests, and tolerance to a wide range of abiotic conditions [41–43]. While modifying lignin content through transgenic approaches provides a promising route for improving saccharification efficiency and ethanol production, information regarding the impact of such modifications on the ability of these feedstocks to tolerate biotic and abiotic stresses when grown under natural environmental conditions is limited. This is an important consideration since the commercial viability of low-lignin bioenergy feedstocks will depend on their growth and fitness in an agronomic setting, which could be enhanced or debilitated from pleiotropic effects of lignin modification. This review will provide an overview of efforts to characterize the effects of lignin pathway modifications on phenylpropanoid metabolism and plant stress-related processes, including what has been learned from studies in model plants, as well as recent knowledge gained from studies with species that have been identified as potential bioenergy feedstocks. Since most of these studies have been performed in controlled greenhouse environments, the possible implications of altered stress-related metabolism for the field performance of low-lignin bioenergy feedstocks will also be explored in this review.

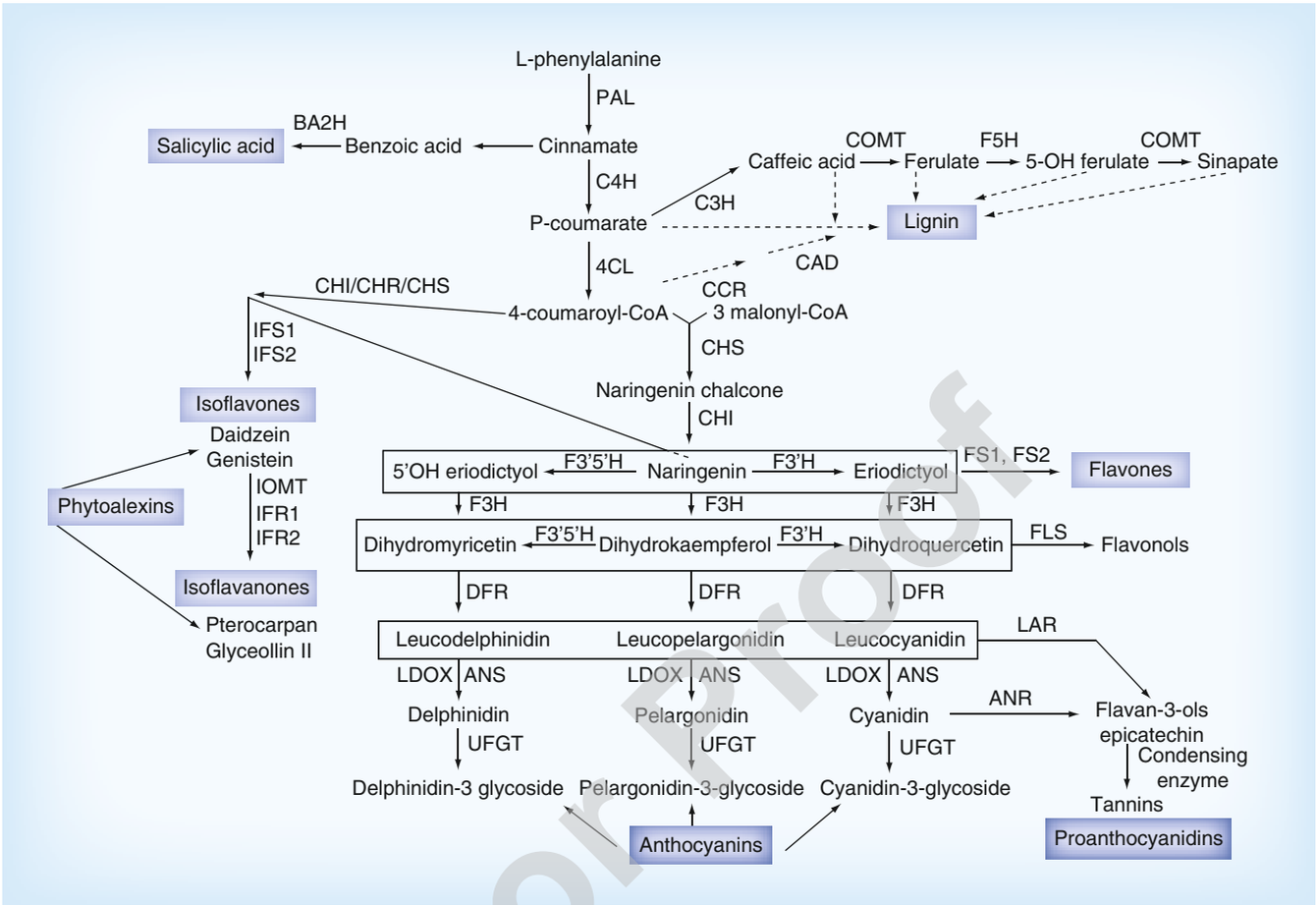

Figure 1. The phenylpropanoid metabolic pathway.
4CL: 4-coumarate:CoA ligase; ANR: Anthocyanidin reductase; ANS: Anthocyanidin synthase (also called LDOX: Leucoanthocyanidin dioxygenase); BA2H: Benzoic acid 2-hydroxylase; C3H: *P*-coumarate 3 hydroxylase; C4H: Cinnamate 4-hydroxylase; CAD: Cynnamyl alcohol dehydrogenase; CCR: Cynnamoyl CoA reductase; CHI: Chalcone isomerase; CHR: Chalcone reductase; CHS: Chalcone synthase; COMT: Caffeic O-methyltransferase; DFR: Dihydroflavonol-4-reductase; F3'H: Flavonoid 3'-hydroxylase; F3',5'H: Flavonoid 3',5'-hydroxylase; F3H: Flavanone 3-hydroxylase; F5H: Ferulic acid 5-hydroxylase; FLS: Flavonol synthase; FS: Flavone synthase; IFR: Isoflavone reductase; IFS: Isoflavone synthase; IOMT: Isoflavone O-methyltransferase; LAR: Leucoanthocyanidin reductase; PAL: Phenylalanine ammonia-lyase; UFGT: UDP-flavonoid glucosyltransferase.
Reproduced from [30].

Alterations to phenylpropanoid metabolism

Phenylpropanoid metabolism in plants is an intricate network of pathways responsible for the synthesis of a broad range of secondary metabolites including flavonoids, isoflavonoids, sinapate esters and lignin. Intermediate and end products of phenylpropanoid metabolism function as antimicrobial and antiherbivory compounds, antioxidants, pigments and UV-protectants [29,44]. The compounds that specifically aid in plant responses to stress range from simple precursor metabolites such as hydroxycinnamic acids, to more complex compounds such as flavonoids, isoflavonoids and stilbenes [45]. Phenylpropanoid metabolic pathways can be developmentally induced in specific tissues, or activated in response to biotic and abiotic stress. Initiation of the phenylpropanoid pathway begins with the conversion of phenylalanine, an amino acid product of the shikimate pathway, to cinnamic acid via phenylalanine ammonia-lyase (Figure 1; [30]). The next two steps are catalyzed by cinnamate 4-hydroxylase and 4-coumaroyl CoA ligase to produce *p*-coumaroyl CoA, a metabolite that serves as a branch point from which other metabolic pathways in the phenylpropanoid network diverge [44]. The enzymes and enzyme families that control metabolic channeling among the major classes of phenylpropanoid products have been discussed in reviews [44,46], as well as the **transcription factors** and signaling networks that regulate gene expression among the various secondary metabolic pathways [47,48].

Key term

RNAi-mediated gene silencing: RNAi methods are used to block the translation of a specific target gene into its protein product, thus preventing it from becoming functional. In lignin genetic engineering, this approach can be used to reduce the expression levels of lignin biosynthetic genes.

As the second most abundant natural polymer after cellulose, lignin is a major end product of phenylpropanoid metabolism. The lignin-specific branch of the phenylpropanoid pathway is responsible for the synthesis of *p*-coumaryl, coniferyl and sinapyl alcohol monolignols. These monolignols serve as precursors for the production of *p*-hydroxyphenyl, guaiacyl and syringyl monomeric lignin subunits, respectively, which undergo polymerization by peroxidases to form the lignin polymer [49]. More recently, the discovery of a cachetyl lignin polymer (C-lignin), comprised solely of caffeyl alcohol monomers, has been observed in the seed coats of some species [50]. While many of the enzymes that participate in lignin biosynthesis can independently utilize multiple compounds as substrates, mixed substrate reactions reveal strong enzyme preferences for specific substrates and a high degree of competitive interactivity among enzymes for particular substrates [51–53]. This gives rise to a main metabolic stream toward the production of monolignols, with each substrate being primarily catalyzed by a specific enzyme **(Figure 2)** [4,54]. Characterization of mutant and transgenic plants with altered lignin biosynthesis has helped elucidate the influence of individual enzymes on lignin content and composition [49,55].

While the effects of altered lignin biosynthesis on total lignin content and lignin polymer composition have been studied in great detail, the consequences of lignin pathway perturbations on other metabolic processes are not as well understood. Recent transcriptomic, proteomic and metabolomic profiling of such plants have helped reveal some of these broader effects. Several studies have indicated that downregulating single or multiple lignin pathway genes can alter the metabolic flux through the phenylpropanoid pathway, and differentially affect the biosynthesis of other secondary compounds **(Table 1)**. Depending on the lignin biosynthetic gene(s) being suppressed, plants can undergo various metabolic changes in response to the reduced carbon flow into lignin biosynthesis. In the next sections, the known biochemical effects of altering the expression of characterized lignin biosynthetic genes will be examined.

▪ Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase

Hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT) is a member of a large gene family encoding for acyltransferases, a group of enzymes that catalyze the acylation of secondary metabolites. HCT catalyzes an early step in the phenylpropanoid pathway and initiates the carbon flux toward the monolignol-specific branches by catalyzing the conversion of *p*-coumaroyl-CoA into *p*-coumaroyl shikimate [56]. *P*-coumaroyl-CoA is a metabolite located at a branching point in the phenylpropanoid pathway, where the monolignol and flavonoid biosynthetic pathways diverge. If catalyzed by HCT, *p*-coumaroyl-CoA is converted into shikimate and quinate esters, and carbon is directed toward the synthesis of monolignols [56,57]. Alternatively, *p*-coumaroyl-CoA can also serve as a substrate for chalcone synthase (CHS), an enzyme that catalyzes the initial step toward flavonoid biosynthesis. If catalyzed by CHS, the metabolic flux will instead be directed toward the production of flavonoids, including flavonols, anthocyanins and tannins [58,59]. Thus, competition between HCT and CHS for the same substrate makes these two enzymes highly influential in controlling the metabolic flux toward either monolignol or flavonoid biosynthesis.

Lignin downregulation by **RNAi-mediated gene silencing** of HCT in *A. thaliana* resulted in a hyperaccumulation of flavonoid products [32]. The observed increase in flavonoid biosynthesis was suggested to be a consequence of the metabolic flux being redirected away from the lignin pathway in the absence of HCT, and into the flavonoid pathway through CHS activity. High performance liquid chromatography profiling revealed that flavonoid products, most notably flavonols and anthocyanins, accumulated in higher amounts in the HCT-downregulated plants compared with control plants. HCT-downregulated plants also exhibited a dark purple coloration of the leaves relative to the controls as a result of the anthocyanin accumulation. Similarly, downregulation of HCT in alfalfa (*M. sativa* L.) also resulted in an increase in flavonoid biosynthesis and an accumulation of anthocyanins, which were suggested to be a result of metabolic spillover from suppressed lignin biosynthesis into the flavonoid pathway [31]. In addition to evidence supporting a metabolic spillover into flavonoid metabolism, the expression of several flavonoid biosynthetic pathway genes were induced in the HCT-silenced plants; the activation of these genes could also be contributing to the observed flavonoid accumulation. A significant increase in coumaric acid was also observed in HCT-silenced plants; researchers hypothesized that the accumulating HCT substrate, *p*-coumaroyl-CoA, may either undergo hydrolysis into coumaric acid or be redirected into the flavonoid biosynthetic pathway.

▪ *P*-coumarate 3-hydroxylase

After being synthesized by HCT, *p*-coumaroyl shikimate is converted into caffeoyl shikimate by *p*-coumarate 3-hydroxylase (C3H) [60,61]. Downregulation of C3H in hybrid poplar (*Populus grandidentata* × *Populus alba*) resulted in the accumulation of soluble secondary

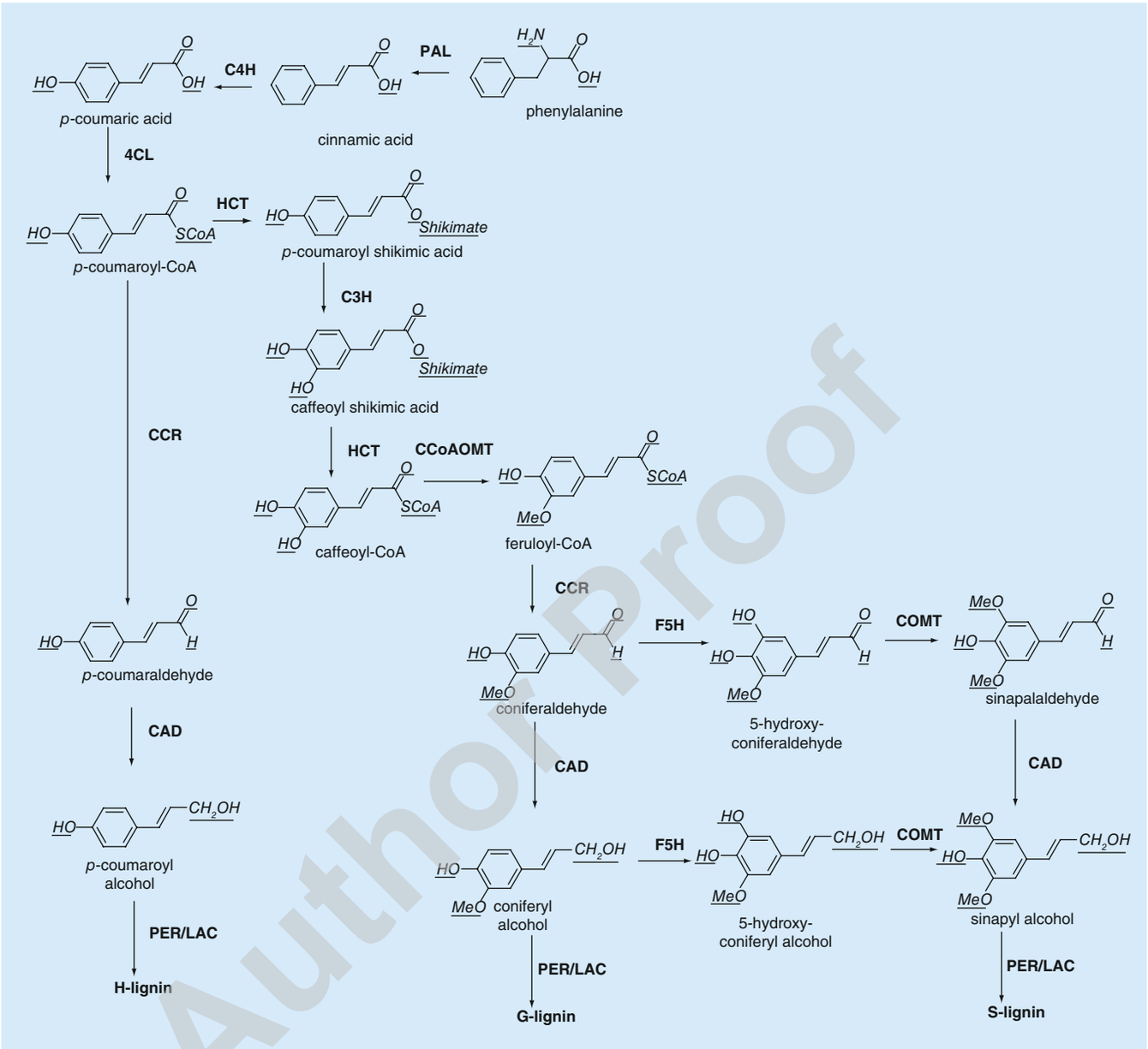

Figure 2. One of the current views of the lignin biosynthetic pathway. The enzymes involved in the pathway are: 4CL: 4-coumarate-CoA ligase; C3H: Coumarate 3-hydroxylase; C4H: Cinnamate 4-hydroxylase; CAD: Cinnamyl alcohol dehydrogenase; CCoAOMT: Caffeoyl CoA 3-*O*-methyltransferase; CCR: Cinnamoyl-CoA reductase; COMT: Caffeic acid 3-*O*-methyltransferase; F5H: Ferulate 5-hydroxylase; HCT: Hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyl transferase; LAC: Laccase; PAL: Phenylalanine ammonia lyase; PER: Peroxidase.

metabolites, the majority of which were *p*-coumaric acid-derived phenylglucosides, including *O*-*p*-coumaroyl-β-D-glucoside and populoside, grandidentatin and trichocarposide. The researchers hypothesized that the decreased C3H activity could cause *p*-coumarate to be diverted into ester-linked glucosides, which would prevent a potentially toxic accumulation of this substrate by allowing it to be mobilized to the phloem [33].

▪ Caffeoyl-CoA O-methyltransferase

Caffeoyl-CoA O-methyltransferase (CCoAOMT) methylates caffeoyl-CoA to feruloyl-CoA, as well

Table 1. Summary of the effects of altered lignin biosynthesis in transgenic or mutant plants on the synthesis of phenylpropanoid-derived secondary metabolites.

Gene	Type of modification	Plant	Effect on phenylpropanoid metabolism	Ref.
HCT	Downregulation	*Arabidopsis thaliana*	↑ flavonoids	[32]
	Downregulation	Alfalfa	↑ flavonoids	[31]
			↑ coumaric acid	[31]
C3H	Downregulation	Poplar	↑ phenolic glucosides	[33]
CCoAOMT	Downregulation	Poplar	↑ phenolic glucosides	[64]
	Downregulation	Alfalfa	↑ caffeoyl glucosides	[65]
CCR	Mutant	*A. thaliana*	↑ feruloyl malate	[69]
			↓ sinapoyl malate	[69]
	Downregulation	Poplar	↑ ferulic acid	[40]
			↑ sinapic acid	[40]
	Downregulation	Tobacco	↑ ferulic acid	[39,71]
			↑ sinapic acid	[39,71]
COMT	Mutant	*A. thaliana*	↓ sinapoyl malate	[36,73]
			↑ hydroxyferuloyl malate	[36,73]
	Downregulation	Switchgrass	↑ ferulic acid	[74]
			↑ 5-hydroxyferulic acid	[74]
			↑ vanillin	[74]
			↑ 5-hydroxyconiferaldehyde	[74]
			↑ iso-sinapyl alcohol	[74]
CAD	Downregulation	Switchgrass	↑ chlorogenic acid	[17]
	Downregulation	Flax	↑ ferulic acid	[75]
			↑ *p*-coumaric acid	[75]
	Downregulation	Tobacco	↑ syringic acid	[76]
			↑ ferulic acid	[76]
			↑ *p*-coumaric acid	[76]
			↑ sinapic acid	[76]
ZmMYB42 transcription factor	Overexpression	*A. thaliana*	↓ flavonoids	[38]
			↓ sinapoyl malate	[38]
ZmMYB31 transcription factor	Overexpression	*A. thaliana*	↑ flavonoids	[34]
			↓ sinapoyl malate	[34]

↑: Accumulation; ↓: Suppression.

as 5-hydroxyferuloyl-CoA to sinapoyl-CoA. This enzyme, along with caffeic acid 3-*O*-methyltransferase (COMT), is involved in catalyzing the methylation of the monolignol precursors [62,63]. Downregulation of CCoAOMT in poplar resulted in a redirection of the metabolic flux away from monolignol biosynthesis, and into the pathway leading from caffeic acid to sinapic acid. The increased flux into this pathway resulted in an increased synthesis and accumulation of phenolic acid glucosides in transgenic plants relative to controls, including O3-β-D-glucopyranosyl-caffeic acid, O 4-β-D-glucopyranosyl-vanillic acid and O 4-β-D-glucopyranosyl-sinapic acid [64]. Similarly, downregulation of CCoAOMT in alfalfa resulted in an accumulation of caffeoyl glucosides [65].

▪ Cinnamoyl-CoA reductase

Cinnamoyl-CoA reductase (CCR) catalyzes cinnamoyl-CoA esters to their corresponding cinnamaldehydes, which is considered to be the first step in the monolignol-specific branches of the lignin biosynthetic pathway [66–68]. For most species, the predominant role of CCR in lignin biosynthesis is the reduction of feruloyl-CoA into coniferaldehyde [49]. In *A. thaliana*, poplar and tobacco, suppressed CCR activity has been shown to affect the soluble phenolics composition, most notably by increasing the accumulation of various ferulate derivatives. In studies with *A. thaliana* mutants deficient in CCR, a decreased flow of metabolites into lignin biosynthesis was accompanied by a reduction in sinapoyl malate biosynthesis, and an increased flow toward the synthesis of feruloyl malate, as well as an incorporation of ferulic acid into the lignin polymer [69]. Two possible mechanisms employed by plants to avoid the accumulation of monolignol precursors is by sequestering them into storage metabolites or incorporating them into the cell wall structure [70]. It was hypothesized that the CCR-deficient mutants avoid a potentially toxic buildup of feruloyl-CoA, the preferred substrate of CCR, via one or both the following

redirection mechanisms: by hydrolyzing feruloyl-CoA into ferulic acid, which is further processed into feruloyl malate; or by transferring feruloyl-CoA-derived ferulic acids to the cell wall and incorporating them into the lignin structure [69]. Transgenic poplar and tobacco plants with CCR-downregulation exhibited modifications to the soluble phenolics composition similar to those observed in *A. thaliana* CCR mutants [39,40,71]. In poplar, CCR downregulation was associated with an overall increase in the soluble phenolics content, most notably in the levels of ferulic and sinapic acid esters, relative to control plants [40]. In addition, the concentration of ferulic acid in transgenic tissues increased relative to coniferaldehyde. It was suggested that the decreased conversion of feruloyl-CoA into coniferaldehyde in CCR-downregulated plants caused a diversion of the metabolic flow away from lignin biosynthesis, which was compensated for by an increased flow toward the synthesis of ferulic acids. Similar to that observed in the CCR mutants of *A. thaliana*, NMR and thioacidolysis analyses of CCR-downregulated poplar indicated that some of the accumulating ferulic acid was transported to the cell wall and cross-coupled with lignin. Transgenic tobacco with suppressed CCR activity also exhibited an increased metabolic flux toward the production of ferulic acid and sinapic acid relative to control plants [39,71]. In one of these studies, an increase in glycosylated and quinylated derivatives of feruloyl-CoA was observed in transgenic plants relative to their controls [39]. It was proposed that the accumulating feruloyl-CoA could be quinylated or redirected toward the synthesis of ferulic acid. The ferulic acid could then be partially converted to sinapic acid and detoxification of these acids could occur via glycosylation. Increased transcript levels of 3-deoxy-D-arabino-heptulosonate-7-phosphate, a regulator of the carbon flux into shikimate metabolism, indicated that the metabolic flux may also be partially redirected toward the shikimate pathway.

▪ Caffeic acid 3-*O*-methyltransferase

COMT is a member of the *O*-methyltransferases, a family of enzymes that catalyze the methylation of various secondary metabolites in the phenylpropanoid pathway [72]. In lignin biosynthesis, COMT is primarily responsible for catalyzing the *O*-methylation of the 5-hydroxyl group of 5-hydroxyconiferaldehyde to produce sinapaldehyde [37]. A suppression of COMT enzyme activity has been shown to affect the phenolics profiles of *A. thaliana*, tobacco and switchgrass [36,37,73,74]. *A. thaliana* mutants deficient in COMT activity accumulate significantly less sinapoyl malate relative to controls [36,73]. One of these reports also indicated a significant increase in hydroxyferuloyl malate in COMT mutants compared with controls; this compound is a derivative of the COMT substrate, 5-hydroxyconiferaldehyde [36]. In switchgrass, RNAi-mediated gene silencing of COMT resulted in an accumulation of phenolic acids and aldehydes, most notably in ferulic acid, 5-hydroxyferulic acid and ferulic acid-glycoside conjugates, vanillin and 5-hydroxyconiferaldehyde. Additionally, a novel monolignol-like metabolite was observed in the transgenic plants, identified as trans-3, 4-dimethoxy-5-hydroxycinnamyl alcohol (iso-sinapyl alcohol) [74].

▪ Cinnamyl alcohol dehydrogenase

Cinnamyl alcohol dehydrogenase (CAD) catalyzes the last step in the monolignol-specific pathway by reducing cinnamaldehydes into their corresponding cinnamyl alcohols [68]. In switchgrass, downregulation of CAD resulted in a 40–170% increase in the level of chlorogenic acid (caffeoyl quinic acid) in most of the transgenic lines relative to the control [17]. Downregulation of CAD in flax (*Linum usitatissimum* L.) led to an increase in total phenolic content, including a twofold increase in ferulic acid and 30% increase in *p*-coumaric acid compared with the control plants [75]. In CAD-downregulated tobacco, an increased metabolic flux toward the production of soluble phenolics was also observed, particularly in levels of syringic, ferulic, *p*-coumaric and sinapic acids [76]. Additionally, ferulic acid increased by 58-fold in the transgenic plants relative to the wild-type controls. Another study with CAD-downregulated tobacco reported an increase in the accumulation of the two substrates of CAD, coniferaldehyde and sinapaldehyde, in the transgenic plants relative to controls [39]. Additionally, it was suggested that CAD downregulation may also result in a partial flux toward the shikimate pathway, as indicated by increased transcript levels of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase [39].

▪ Myeloblastosis transcription factors

Rather than silencing the expression of individual lignin biosynthetic genes, an alternative approach for decreasing lignin in cell walls is to manipulate the activity of transcription factors that act as repressors of lignin genes. Myeloblastosis (MYB) proteins comprise a diverse family of transcription factors that play important regulatory roles in various plant functions. The R2R3-MYB family of transcription factors has been described as playing an important role in the regulation of lignin biosynthetic genes [5]. The activities of R2R3-MYB transcription factors are generally not restricted to lignin biosynthesis, but rather have an influence on gene expression in multiple pathways within the phenylpropanoid network, including phenolics, anthocyanins, lignins

Key term

Pathogenesis-related proteins: Play a general role in plant response and adaptation to biotic stresses, typically induced by the host plant in response to viral, bacterial or fungal infections.

and flavonols [77]. *A. thaliana* plants with over-expression of ZmMYB42, an R2R3-MYB transcription factor derived from maize (*Zea mays*), exhibited a suppression of several lignin biosynthetic genes and a corresponding decrease in total lignin content relative to control plants [38]. Additionally, these plants were reported to have a 66% reduction in total phenolic content, mainly from a significant reduction in flavonols. Suppression of flavonoid biosynthesis was attributed to the negative regulation of ZmMYB42 on two major flavonoid pathway genes. In addition to flavonoid and lignin suppression, these plants also exhibited lower levels of sinapoyl malate compared with controls. This reduction in sinapoyl malate biosynthesis could be related to the negative regulation of ZmMYB42 on the expression of cinnamate 4-hydroxylase, ferulate 5-hydroxylase and COMT, as a reduced expression of these genes in mutant plants has been associated with a decrease in sinapoyl malate synthesis [73,78]. Additionally, this reduction could also be explained by the negative regulation of ZmMYB42 on aldehyde dehydrogenase, the enzyme catalyzing the initial step toward sinapoyl malate biosynthesis [38]. *A. thaliana* plants expressing another R2R3-MYB transcription factor, ZmMYB31, also exhibited a significant decrease in lignin content and a reduction in sinapoyl malate biosynthesis relative to control plants [34]. However, in contrast to the ZmMYB42 plants, there was an increase in flavonoid biosynthesis in ZmMYB31 over-expressing plants relative to controls. This increase in flavonoid biosynthesis was accompanied by an accumulation of anthocyanins in transgenic tissues. The researchers concluded that the over-expression of this transcription factor in *A. thaliana* results in a reduced flux of carbon into lignin and sinapoyl malate biosynthesis, and a redirection of the flux into flavonoid biosynthesis [34].

Effects of lignin modification on plant stress

As discussed above, suppressing single or multiple lignin biosynthetic steps can lead to the accumulation or suppression of other secondary metabolites. Many of these compounds play important roles in plant–environment interactions, and altering their biosynthesis can influence the susceptibility of plants to stresses. Additionally, transcriptomic and proteomic studies have indicated that lignin modifications can trigger an increase in the expression of stress response-related genes [31,34,39,40], and in one report there was a broad upregulation of defense-related genes that was associated with an enhanced tolerance to biotic and abiotic stresses [31]. The exact mechanisms triggering changes in gene expression in lignin-modified plants are not yet clearly understood. The reduction in lignin content may trigger signaling pathways that cause the activation of stress genes [34]. One hypothesis is that the activation of stress-related genes could be triggered by changes to the structural organization of the secondary cell wall, either by mimicking cell wall wounding or pathogen damage [39,40], or by facilitating the release of primary cell wall polysaccharide components that act as elicitors of defense responses [31]. In addition, there is accumulating evidence that altering cell wall integrity can induce long-term modifications to gene expression, including the constant activation of defense-related genes [79,80].

▪ Biotic stress-related effects

Several phenolic compounds play essential roles in mediating plant–microbe interactions [81], and modifications to the phenolic profile resulting from altered phenylpropanoid metabolism have been shown to influence such interactions in mutant and transgenic plants with suppressed lignin biosynthesis. Hydroxyferuloyl malate, a derivative of the COMT substrate, accumulated in *A. thaliana* mutants deficient in COMT activity [36]. Elevated levels of this compound were correlated with an enhanced resistance to the downy mildew-causing oomycete pathogen, *Hyaloperonospora arabidopsidis*, and an inhibition of asexual sporulation was observed in the COMT mutant plants compared with wild-type controls. *In vitro* assays showed that application of hydroxyferuloyl malate to the oomycete pathogen enhanced sexual reproduction and weakened mycelium vigor [36]. Additionally, transgenic tobacco plants with downregulated COMT activity developed significantly smaller tumors than control plants when inoculated with *Agrobacterium tumefaciens*, a soil bacterium that causes crown gall disease. These plants had lower *Agrobacterium* virulence (*vir*) gene-inducing activities and lower levels of soluble phenolics including acetosyringone, a known elicitor of *vir* expression in *Agrobacterium* [37].

An upregulation of genes encoding for **pathogenesis-related proteins** and other proteins involved in plant defense toward pathogens has been observed in poplar, *A. thaliana* and alfalfa plants with modified lignin biosynthesis. In CCR-downregulated poplar, increased transcript levels of a U-box domain protein resembling the CMPG1 protein triggered by fungal elicitors in *Petroselinum crispum* were observed relative to control plants [40]. In *A. thaliana* plants over-expressing the ZmMYB31 transcription factor, a proteome analysis showed increased levels of osmotin relative to control plants [34]; osmotin is a pathogenesis-related (PR) protein that primarily functions as a plant defense protein by providing resistance against a variety of fungal pathogens [82]. Downregulation of HCT in alfalfa

resulted in a constitutive activation of defense responses, and a transcriptome analysis of stem tissue revealed that the expression of various PR transcripts was 5- to 56-fold higher in transgenic plants than in controls [31]. It was suggested that this constitutive PR gene expression could be related to the altered cell wall integrity. Fragments of pectin, a galacturonic acid-rich component of plant primary cell walls, were shown to be more easily released from the secondary cell wall in transgenic alfalfa compared with controls [31]. Previous studies have shown that galacturonides can trigger defense responses in several plant species [83–85]. In addition, these plants had higher levels of salicylic acid (SA) relative to controls, a biotic defense-related phytohormone that has previously been shown to stimulate the upregulation of PR proteins in response to infection and enhance resistance to a wide range of pathogens [31,86]. Levels of SA were correlated with the amount of extractable pectin in the cell wall. SA can be synthesized either from cinnamate by phenylalanine ammonia-lyase activity, or from chorismate by isochorismate synthase activity, and the production of SA through the isochorismate pathway has been observed in *A. thaliana* plants during defense response [87]. Therefore, it was hypothesized that the continuous leaching of these pectic elicitors from the cell wall as a result of reduced lignification could trigger the production of SA through the isochorismate pathway, and high levels of SA could then stimulate the production of PR proteins responsible for the observed constitutive defense response [88]. Since constitutive expression of PR proteins in plants has previously been shown to confer resistance to a wide range of pathogens [89,90], transgenic alfalfa plants were exposed to alfalfa anthracnose (*Colletotrichum trifolii*) to test whether elevated levels of PR transcripts would translate into enhanced pathogen resistance. Incidence and severity of necrotic legions were significantly lower in HCT-downregulated plants compared with the controls [31].

▪ Abiotic stress-related effects

Oxidative stress, photorespiration & UV sensitivity

Lignin modifications have been shown to be associated with the activation of genes involved in oxidative stress responses in tobacco, poplar and *A. thaliana*. In tobacco, transgenic plants had elevated transcript levels of metallothionein and glutathione S-transferase, two enzymes involved in detoxifying oxidative stress metabolites, along with heat shock transcription factors that promote transcription of genes involved in protection against oxidative stress damage [39,91,92]. In addition to oxidative stress-related transcripts, an increase in the abundance of photorespiratory-related transcripts and metabolites were observed in transgenic plants relative to controls, and gas-exchange analyses confirmed that the transgenic plants exhibited elevated photorespiration. This elevated photorespiration was suggested to be caused by an enhanced efficiency of photosystem II (PSII), the first protein complex in the light-dependent reactions of photosynthesis. Gas-exchange analyses indicated that the increased efficiency of PSII in the transgenic plants was not associated with a change in photosynthetic CO_2 assimilation. Therefore, researchers hypothesized that the transgenic plants absorbed more light energy than could be used for photosynthesis, and the observed increase in photorespiration could be a protective mechanism against light-induced damage to the photosynthetic apparatus [39]. An accumulation of H_2O_2 was also observed in the leaves of the CCR-downregulated plants, and lesions developed that resembled those observed in catalase-deficient tobacco grown under high-light conditions. It was hypothesized that photorespiration, specifically the elevated levels of photorespiratory H_2O_2, could be triggering the oxidative stress phenotype. An alternative hypothesis for the observed oxidative stress phenotype suggested that the altered cell wall structure could be actively inducing the expression of oxidative stress-related genes as part of a wound-like response. In support of this hypothesis, metabolomic profiling showed an increase in the levels of feruloyl tyramine in CCR-downregulated plants compared with the controls [39]. In solanaceous plants, feruloyl tyramine has been shown to accumulate at the site of wounding or pathogen attack in order to reinforce the cell wall [93]. Similar to that observed in tobacco, CCR-downregulation in poplar appeared to trigger a wound-like stress response and a corresponding increase in the expression of oxidative stress-related transcripts, also hypothesized to be induced by a defective cell wall [40]. Additionally, these plants had significantly higher levels of transcripts encoding for a PSII reaction center protein and a glutamine synthetase compared with controls, indicating a possible connection between oxidative stress and photorespiration similar to the one described in CCR-downregulated tobacco [40]. In *A. thaliana*, proteome analyses indicated that plants with over-expression of ZmMYB31 had increased levels of enzymes involved in protecting against oxidative stresses compared with control plants. ZmMYB31 also downregulates the expression of aconitase, an enzyme that plays a role in mediating oxidative stress and associated cell death, and it has been reported that mutant *A. thaliana* plants deficient in this enzyme are more tolerant to oxidative stress [34,94].

The suppression of lignin biosynthesis has also been shown to affect the production of secondary metabolites that function as UV protectants. UV light-induced stress can induce the synthesis of flavonoids in epidermal tissues of plants, where they function as a protective screen against UV damage and subsequent cell death

by absorbing light in the UV-B range [59,95]. Sinapoyl malate and other sinapate esters have also been recognized as important UV protectants in *A. thaliana* [95]. In CCR-deficient mutant plantlets of *A. thaliana*, a substantial reduction in sinapoyl malate biosynthesis was observed relative to control plants [69]. Exposure to UV light resulted in slower growth of the mutant plantlets relative to controls, and their leaves were yellow, indicating an increased sensitivity to UV light. Similarly, ZmMYB42 over-expression in *A. thaliana* resulted in the downregulation of several phenylpropanoid pathway genes including those involved in sinapoyl malate and flavonoid biosynthesis [38]. As a result, transgenic plants were more sensitive to UV light, as indicated by a yellowing of the leaves relative to control plants [38]. *A. thaliana* plants with over-expression of another MYB transcription factor, ZmMYB31, also had lower levels of sinapoyl malate compared with controls [34]. However, unlike the ZmMYB42 plants, ZmMYB31 over-expression resulted in an increase in levels of UV-protecting flavonoids. Despite this increase in flavonoids, transgenic *A. thaliana* plants were still highly sensitive to UV radiation compared with controls, displaying an upward leaf curling phenotype resembling those observed in mutant plants deficient in UV-protectant compounds [34,95]. The researchers concluded that these results, in addition to previous studies involving mutant plants, suggest that sinapate esters may play a more important role than flavonoids in protecting *A. thaliana* against UV radiation [34].

Water stress

In HCT-downregulated alfalfa, transcriptome profiling revealed an upregulation of abiotic stress-related transcripts, the majority of which were heat and drought stress-related [31]. Metabolomic analyses of transgenic plants also showed elevated levels of abscisic acid, a phytohormone that plays a central role in sensing water deficiency and activating the expression of drought stress-related genes [31,96]. To investigate whether the observed changes in drought stress-related gene expression were associated with an enhanced tolerance to drought, plants were deprived of water for a period of 9 days. Transgenic plants exhibited fewer symptoms of drought stress than controls and were able to recover completely after 5 days of rehydration, while control plants showed extensive damage and did not survive. Leaf water potential measurements showed that water potential in the transgenics decreased at a slower rate than the controls throughout the experiment. Additionally, changes that are commonly associated with adaptation to drought stress, including reduced leaf transpiration rate and increased number of stomatal cells, were observed in transgenic leaf tissue [31,97].

Future perspective

The use of systems biology-based approaches that incorporate information at the transcript, protein and metabolite level are continually improving our understanding of how plants respond to altering lignin. It is important to examine and understand these broader effects of lignin modification since the commercial viability of altered-lignin bioenergy feedstocks will depend not only on improved biofuel traits, but also on their ability to perform at least as well as their nontransgenic counterparts in the field. This performance includes optimal growth potential in the absence of stress, the ability to tolerate abiotic stresses and the ability to resist pests. Vulnerability on any of these fronts would likely make modified feedstocks an undesirable risk to farmers. Therefore, field-based experiments to characterize lignin-modified feedstocks in these crucial areas are absolutely required.

To date, most of the research with transgenic low-lignin feedstocks grown in the field has been done in trees and forage crops for evaluating traits related to improved pulping or digestibility, respectively [6,98]. More recently, saccharification efficiency has been assessed in field-grown bioenergy crop species [21,23,99]. Such studies have provided valuable insight into the impacts of altered lignin content on susceptibility to biotic and abiotic stresses in a field setting. In a 4-year field evaluation of poplar plants with reduced lignin content for improved pulping performance, there was no effect of lignin modification on the susceptibility to insect herbivory or rust (*Melampsora* sp.) [6]. Similarly, field-grown COMT-downregulated sugarcane was not more susceptible to orange rust (*Puccinia kuehnii*) compared with nontransgenic controls [21]. On the other hand, COMT-downregulation in field-grown perennial ryegrass plants led to an increased susceptibility to rust (*Puccinia* species) relative to control plants [100]. In addition to examining biotic stress interactions in lignin-modified crops, abiotic stress-related effects have also been assessed in the field. A 2-year field study of low-lignin poplar with downregulation of 4-coumaroyl CoA ligase found that the transgenic events with the strongest reduction in lignin content were associated with significantly impaired xylem transport efficiency, resulting in frequent shoot dieback despite being watered regularly [101]. Interestingly, further studies with these plants found that the impaired xylem water conductivity in the lignin-downregulated lines was a result of accumulating phenolics and tyloses that were being deposited in xylem vessels and impeding water transport [102]. In addition to impairment of water transport, the reduction in lignin content in transgenic poplar lines was also associated with decreased wood strength and

stiffness, and an increase in the formation of tension wood [103]. Taken together, the outcomes of these field trials suggest that some types of lignin modifications may be more preferable than others for preserving the growth and viability of low-lignin crops in the field, and this will likely vary from species to species. More extensive field studies are necessary in order to gain a better idea of the types of lignin modifications that will be best tolerated by plants in their environment. Particularly, the indirect effects of modified lignin biosynthesis on secondary metabolism and stress-related gene expression should be further explored under field conditions, as these factors could significantly impact the susceptibility of plants to stresses in an agronomic setting.

In the absence of pertinent field data, we can speculate on the potential agronomic effects of altered stress-related metabolism in low-lignin bioenergy feedstocks. As previously discussed, modified lignin biosynthesis can lead to the differential accumulation or suppression of various phenylpropanoid pathway intermediates, some of which have been shown to influence plant–microbial interactions under controlled environmental conditions. Therefore, it is possible that this altered biochemistry in feedstocks could influence interactions between the plant and bacterial or fungal pests in the field. In particular, levels of lignin precursors such as *p*-coumaric acid, ferulic acid and sinapic acid were shown to accumulate in plants with suppressed lignin biosynthesis; *in vitro*, these metabolites have been shown to have antibacterial and antifungal properties [104]. In switchgrass, the downregulation of CAD led to an accumulation of chlorogenic acid [17]; higher levels of chlorogenic acid have been shown to correlate with increased plant resistance to bacterial pathogens and insect herbivory [105,106]. In addition to influencing plant–microbial interactions, biochemical changes in the plant tissue as a result of altered secondary metabolism could influence the palatability of plants to insects. While the relationship between total lignin content and susceptibility to insect herbivory is unclear [107], a recent field study comparing switchgrass lines with naturally varying lignin levels suggested that other factors, such as the accumulation of compounds that interfere with nutrition, may be more important than overall lignin content in explaining resistance to insect herbivory [108]. In maize, higher levels of *p*-coumaric and ferulic acids were strongly associated with an increased resistance to infestations by maize weevil (*Sitophilus zeamais*) and stem borer (*Sesamia nonagrioides*), respectively, in the field [109,110]. In transgenic poplar with downregulation of C3H, an accumulation of phenolic glucosides was observed. These compounds have previously been shown to deter the activity of fungi and insect pests of poplar, and it was suggested that the elevated levels could potentially enhance defense responses of transgenic poplar toward biotic pests [33]. A constitutive expression of PR genes has also been shown to influence stress interactions, and was shown to increase the tolerance of transgenic alfalfa plants to anthracnose infection [31]; thus, a constitutive activation of biotic defense-related genes could potentially render plants more prepared for defending themselves against pathogens in a field setting.

Modifications to the lignin biosynthetic pathway have also been shown to suppress or enhance levels of secondary compounds that could influence plant acclimation to abiotic stresses. Plants have been shown to accumulate anthocyanins and/or flavonoids as a protective mechanism against salinity stress, UV-B damage, cold temperatures and water stress [111,112]. As previously discussed, downregulation of lignin pathway genes can result in an increased or decreased synthesis of these compounds. Altered levels of these compounds could influence plant sensitivity to abiotic stresses in the field. A field study comparing salt-tolerant and salt-susceptible clones of sugarcane found that the accumulation of soluble phenolics, anthocyanins and flavones were threefold higher in the tolerant clone, and these compounds were suggested to promote salinity tolerance by protecting cytoplasmic structures and chloroplasts from the damages of salinity stress [111]. In addition to their roles in salinity tolerance, levels of anthocyanins and some phenolic acids might play an important role in the acclimation of plants to cold stress, as they have been shown to accumulate in the leaf mesophyll cells in plants exposed to low temperatures [113]. *A. thaliana* with suppressed CCR activity, or with over-expression of MYB transcription factors, were shown to be more sensitive to UV damage due to the consequences of suppressed lignin on sinapoyl malate and/or flavonoid biosynthesis, as previously discussed. A recent study found that UV-B radiation, in addition to inhibiting plant growth, can alter plant tissue biochemistry and result in a significant reduction in cell wall digestibility and the enzymatic release of sugars from the biomass [114]. Although this study was performed in a greenhouse, the results could have significant implications for ethanol production from field-grown low-lignin feedstocks that have been made more susceptible to the effects of UV radiation.

Given this knowledge, it is conceivable that alterations to phenylpropanoid metabolism and/or the upregulation of stress-related genes resulting from lignin modification could positively or negatively influence the agronomic performance of low-lignin

transgenic feedstocks. It is also important to note that there can be significant metabolic costs associated with an increased allocation of energy into the production of defense-related secondary metabolites and constitutive expression of defense-related genes, such that a higher resistance to stresses through either these mechanisms could be associated with stunted growth phenotypes and consequent reductions in biomass yield [115,116]. This potential trade-off between stress resistance and biomass yield should be considered, since high biomass production is also an important trait for bioenergy feedstocks. In conclusion, extensive field evaluations and an improved understanding of the relationship between lignin and stress-related metabolism are necessary to ensure the sustainable growth of transgenic low-lignin bioenergy feedstocks in agronomic field environments.

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Executive summary

Background

- Genetic engineering strategies to reduce total lignin content or modify lignin composition can significantly improve saccharification efficiency and ethanol yield in lignocellulosic feedstocks.
- Recent studies have revealed that lignin pathway modifications can affect various stress-related processes. This review examines the mechanisms underlying these changes and the influences they can have on how plants interact with stresses.

Altered phenylpropanoid metabolism

- The lignin biosynthetic pathway constitutes one of the major branches of the phenylpropanoid pathway. Phenylpropanoid metabolism is responsible for the synthesis of secondary metabolites that play developmental and stress-related roles in plants.
- Suppressing steps along the lignin biosynthetic pathway can alter carbon flow within the phenylpropanoid pathway, leading to the accumulation or suppression of other secondary metabolites.
- The biochemical changes that result from lignin modifications vary depending on which step(s) along the lignin biosynthetic pathway are suppressed.

Effects on plant stress

- Alterations to phenylpropanoid metabolism resulting from lignin downregulation can affect the biosynthesis of secondary compounds that play important roles in plant–environment interactions.
- Lignin modifications can trigger the upregulation of stress-response related genes.
- Experiments in controlled environments demonstrate that changes to these processes can positively or negatively influence the susceptibility of lignin-modified plants to stresses.

Future perspective

- In order to be commercially viable, lignocellulosic feedstocks must perform at least as well as their nontransgenic counterparts in an agronomic field setting. Examining and understanding the broader impacts of altered lignin biosynthesis on stress-related metabolism will help to identify what types of lignin modifications will be best tolerated by the plant without compromising growth and fitness.

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