Altering the Cell Wall and Its Impact on Plant Disease: From Forage to Bioenergy

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Abstract

The individual sugars found within the major classes of plant cell wall polymers are dietary components of herbivores and are targeted for release in industrial processes for fermentation to liquid biofuels. With a growing understanding of the biosynthesis of the complex cell wall polymers, genetic modification strategies are being developed to target the cell wall to improve the digestibility of forage crops and to render lignocellulose less recalcitrant for bioprocessing. This raises concerns as to whether altering cell wall properties to improve biomass processing traits may inadvertently make plants more susceptible to diseases and pests. Here, we review the impacts of cell wall modification on plant defense, as assessed from studies in model plants utilizing mutants or transgenic modification and in crop plants specifically engineered for improved biomass or bioenergy traits. Such studies reveal that cell wall modifications can indeed have unintended impacts on plant defense, but these are not always negative.

INTRODUCTION

Liquid biofuel: a liquid fuel, such as ethanol or isobutanol, generated by fermentation of sugars released from biomass

Recalcitrance: a property of plant cell walls that restricts access to enzymes for deconstruction of cell wall polysaccharides

Hemicellulose: a group of heteropolymeric cell wall matrix polysaccharides, such as xylans and arabinoxylans, present in almost all plant cell walls

Secondary cell wall: a thick layer rich in lignin formed inside the primary cell wall when it has stopped increasing in surface area

Lignin: an abundant phenylpropanoid polymer found primarily in plant secondary cell walls and produced by the oxidative polymerization of *p*-hydroxycinnamyl alcohols (monolignols)

Lignocellulosic biomass drives forage-based milk and meat production and is also emerging as a feedstock for the production of liquid biofuels after processing to release cell wall sugars for fermentation. Limitations (collectively termed recalcitrance) inherent in the properties of lignocellulosic materials negatively impact both forage utilization (49) and processing of biomass for biofuels (53); recalcitrance restricts access of enzymes to the cellulose microfibrils and also probably to hemicelluloses, which together represent the major polysaccharide components of both primary and secondary cell walls (**Figure 1**). These polymers contain the hexose and pentose sugar units that must be released for either animal nutrition (in the rumen) or fermentation to ethanol or other liquid biofuels (in the biofuel refinery). Although the phenomenon of cell wall recalcitrance is complex, the presence of the phenylpropanoid polymer lignin in secondary cell walls contributes a major barrier to cell wall deconstruction (69). This contention is supported by detailed studies of cell wall structures during digestion by fungal and bacterial cellulases (28), as well as by the fact that genetic modification to reduce lignin levels or alter lignin composition results in improved digestibility of forages (21, 49) and in increased soluble sugar and fermentable ethanol yields from bioenergy crops, such as switchgrass (*Panicum virgatum* L.) (41, 106).

Strong cell walls evolved as a critical feature in enabling early plants to colonize the land. As an upright growth habit became necessary to allow plants to outcompete each other for access to sunlight above the foliar canopy, mechanical strength of stems became paramount. It also became critical to maintain hydrophobicity of conducting vessels for efficient transpiration, and lignin, which provides both hydrophobicity and mechanical strength, fulfilled this role admirably. Plants also had to protect their cell walls from penetration by bacterial and fungal pathogens seeking access to the nutrient-rich environment within the cell. Recalcitrance has therefore evolved over millennia to support plant structure and defense. The plant cell wall is dynamic, and both its structure and composition change during pathogenesis, partly as a result of modification by microbial wall–degrading enzymes (28) and partly as a result of induced defenses that are targeted at the wall (124). It therefore seems logical that our attempts to improve plants for agriculture and other industrial purposes might interfere with the outcome of interactions with microbes.

This review addresses the relationships between plant cell wall structure and defense from different but related perspectives. First, we provide a brief overview of the modifications to cell wall structure that appear to function as inducible defense responses. The major part of the review then focuses on the impacts of cell wall modification on defense, covering studies in model systems, such as *Arabidopsis*, and work aimed directly at reducing recalcitrance in forage and bioenergy crops. Much of this information has become available since the last review on the cell wall and plant defense in this series (54). Potential mechanisms linking cell wall modification to plant defense are presented, and recent strategies for balancing reduced recalcitrance with optimal plant performance are outlined.

CHANGES IN THE CELL WALL DURING PLANT DEFENSE

Plant pathogens actively attack cell walls using enzymatic tools (63), and sometimes physical force (118), to gain access to intracellular nutrients. Cell wall–associated plant defense is therefore spatially a first line of defense and is not a static barrier. Rather, plants respond dynamically to pathogen attack at the level of the cell wall through pathogen-triggered lignification (129, 139), deposition of the β1,3-glucan callose (75), structural alterations to cell wall polysaccharides (135), production of reactive oxygen species (65, 90), protein cross-linking (13), and biosynthesis and deposition of antimicrobial compounds (phytoalexins) (3) (**Figure 1**). Many of these features of induced defense are closely associated with formation of papillae within the cell wall at the site of

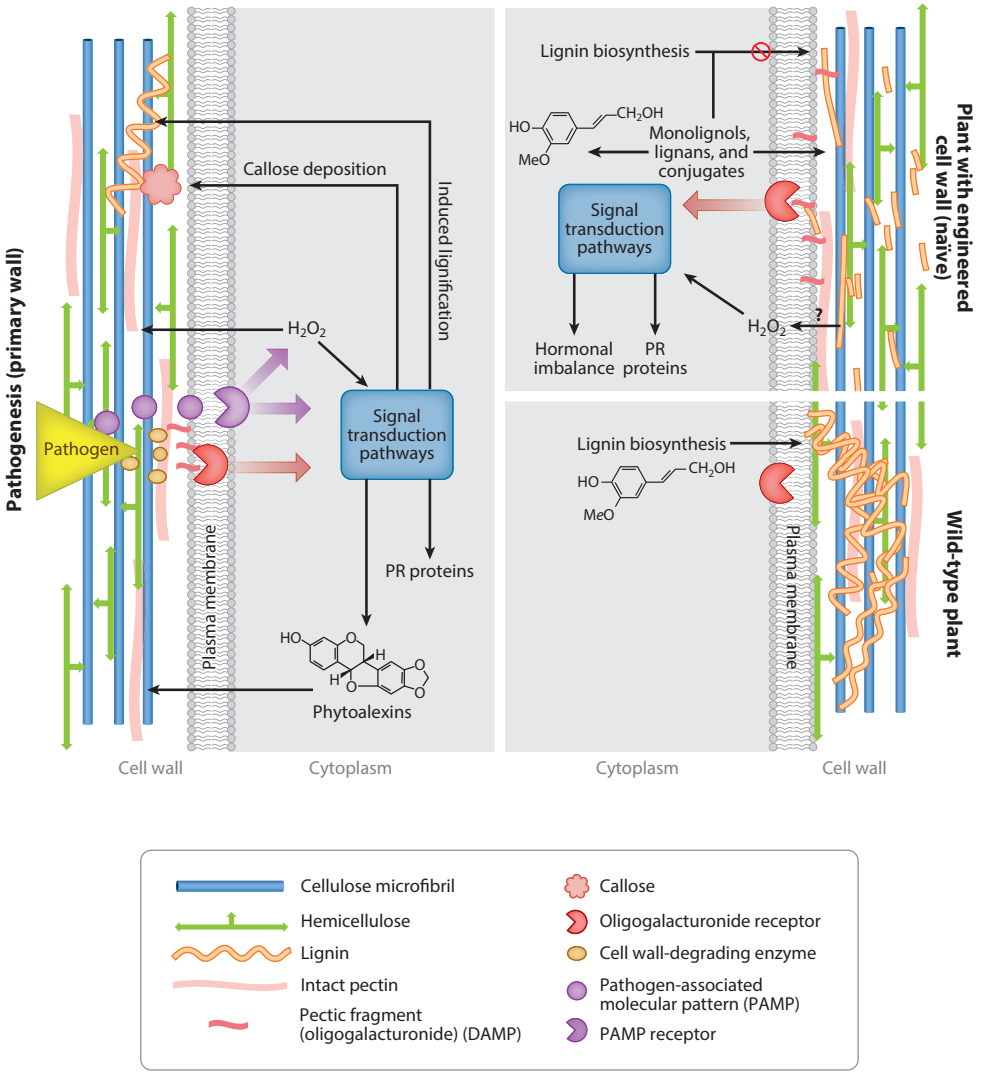


Figure 1

Scheme showing common responses to pathogen infection at the cell wall level and how similar responses may occur in uninfected plants following modifications to cell wall components, particularly lignin. Left panel: Pathogen-induced responses. Right panel (*top*): Constitutive responses in a plant in which lignin levels and degree of polymerization in secondary cell walls have been reduced through mutation or genetic manipulation of a monolignol pathway gene. Significant differences may exist in the responses of different species, or in the same species, dependent on the site of downregulation. Right panel (*bottom*): Wild-type plant. Abbreviations: DAMP, damage-associated molecular pattern; PR, pathogenesis-related. Broad arrows (*red and purple*) represent specific receptor-linked signal pathways; thin black arrows represent downstream biochemical pathways.

attempted penetration (124). Not only do lignins play a role as physical barriers against infection, but the unpolymerized monolignol building blocks of lignins may themselves have antimicrobial activity (5, 7, 54). Furthermore, as discussed in more detail below, cell wall–degrading proteins produced by pathogens can release host cell wall materials (such as oligogalacturonides released from pectin) that may be perceived by plants as signals for the activation of defense responses (19) (**Figure 1**).

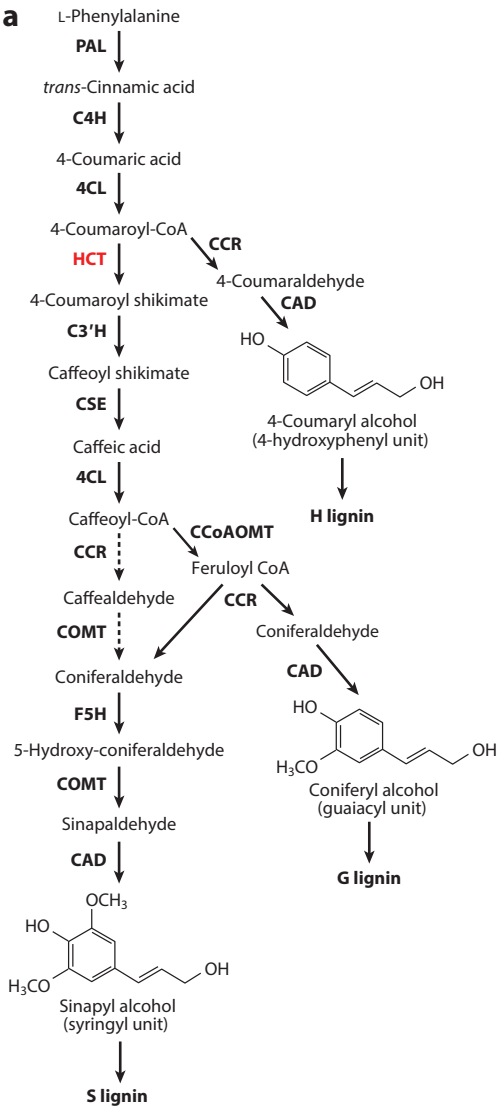
Pectin: a water-soluble polysaccharide from plant cell walls that is rich in uronic acids and can form a gel-like structure

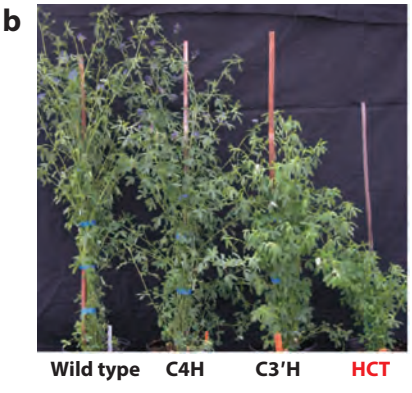
The genes involved in the lignin biosynthesis pathway are now known (11) (**Figure 2*a***) and are often activated after infection by pathogens or elicitor treatment (89). For example, the transcript level of caffeic acid/5-hydroxyconiferaldehyde acid 3-*O*-methyltransferase (*COMT*) (**Figure 2*a***) can be enhanced upon treatment of *Arabidopsis* with flg22, a peptide representing the elicitor-active epitope of bacterial flagellin (144), upon treatment of parsley cells with the effector protein HrpZ (31), or upon treatment of *Arabidopsis* with necrosis-inducing *Phytophthora* protein 1 (NPP1) (35). In fact, some types of lignin biosynthetic genes may be primarily associated with the defense response rather than with developmentally induced lignification. For example, in *Arabidopsis*, cinnamoyl Coenzyme A reductase 1 (*CCR1*) is the major *CCR* gene (**Figure 2*a***) expressed during plant development, whereas *CCR2* is strongly induced in response to bacterial infection (67). A similar situation exists with the multiple *CCR* genes in switchgrass (32). Disruption of the posttranslational protein modification poly(ADP-ribosyl)ation can block lignification along with other innate immune responses, including callose deposition, suggesting that there may be shared mechanisms for the orchestration of multiple cell wall–targeted defense responses (1).

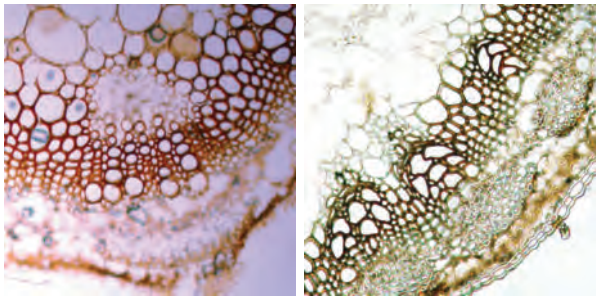
In addition to changes in lignin levels, alteration of lignin monomer composition in response to infection has been described in several different pathosystems, and in some cases different lignin types may be exclusively enriched in response to pathogen attack (34, 46, 51, 66). For example, in wheat leaves inoculated with an elicitor derived from the stem rust fungus *Puccinia graminis* f. sp. *tritici*, lignin content increased but only in syringyl (S) units (**Figure 2*a***) (81). The *Ve* locus–mediated resistance response of tomato to the fungal pathogen *Verticillium dahliae* involves inducible lignification associated with L-phenylalanine ammonia lyase (*PAL*) gene expression (46). Compared with the susceptible lines, the resistant line showed a much faster induction of lignin biosynthesis after inoculation with the pathogen. Guaiacyl (G) and 4-hydroxyphenyl (H) units (**Figure 2*a***) were particularly enriched (46). The resistance of pepper to the same pathogen involves the specific enrichment of H units of lignin in tolerant cultivars (101). After inoculation with the necrotrophic pathogen *Sclerotinia sclerotiorum*, the oilseed crop *Camelina sativa* specifically accumulates G lignin, and this accumulation is associated with the induction of *Camelina sativa*

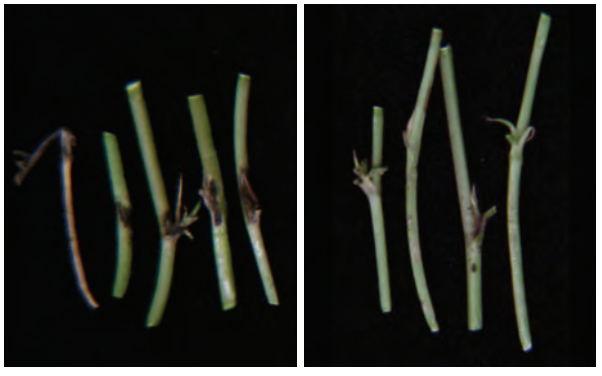
Figure 2

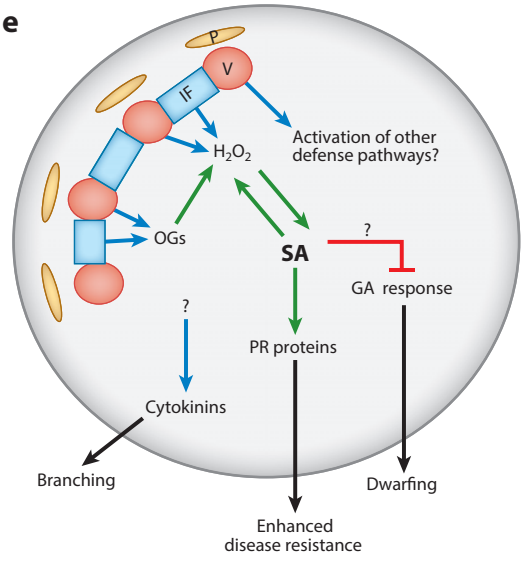
Impacts of antisense-mediated downregulation of hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT; *red*) on growth, vascular development, and disease resistance in alfalfa. (*a*) Biosynthetic pathway to the H, G, and S monolignols. The enzymes are PAL (L-phenylalanine ammonia lyase), C4H (cinnamate 4-hydroxylase), 4CL (4-coumarate:CoA ligase), CCR (cinnamoyl-CoA reductase), CAD (cinnamyl alcohol dehydrogenase), HCT, C3′H (*p*-coumaroyl shikimate 3′-hydroxylase), CCoAOMT (caffeoyl-CoA-3-*O*-methyltransferase), COMT (caffeic acid/5-hydroxyconiferaldehyde 3-*O*-methyltransferase), CSE (caffeoyl shikimate esterase), and F5H (ferulate/coniferaldehyde 5-hydroxylase). Dashed arrows indicate an alternative pathway operating in some species such as *Medicago* (including alfalfa). (*b*) Growth phenotypes of alfalfa plants downregulated in expression of C4H, C3′H, and HCT. Plants were cut back and allowed to regrow in parallel. Photo by author. (*c*) Maule staining (stains S lignin red) of stem cross sections of wild-type and HCT-downregulated alfalfa plants. (*d*) Lesions on stems of wild-type and HCT-downregulated alfalfa plants 18 days after inoculation with *Colletotrichum trifolii*. (*e*) Model integrating genetic and metabolic alterations and phenotypes of HCT antisense alfalfa. Blue arrows represent products of processes (biosynthesis or cell wall leaching), green arrows represent induction, black arrows represent downstream phenotypic responses, and the red line represents repression. Abbreviations: GA, gibberellic acid; IF, interfascicular fiber; OGs, oligogalacturonides; P, phloem; PR, pathogenesis-related; SA, salicylic acid; V, vascular element. Panels *c* and *d* adapted with permission from References 45 and 88.











cinnamoyl Coenzyme A reductase 2 (*CsCCR2*) (34). Interestingly, the constitutive resistance of *Camelina sativa* seems to require a different form of CCR from that involved in developmentally controlled lignification; the transcript level of *CsCCR4* was observed to be more than 10 times higher in the resistant lines than in susceptible lines. Unlike the G-rich lignin induction observed in response to *S. sclerotiorum* infection, the high level of constitutive *CsCCR4* expression in the resistant lines paralleled a high level of constitutive S-lignin deposition (34).

CELL WALL MODIFICATION AND ALTERATION OF PLANT DEFENSE

A significant body of literature indicates that modification of plant cell wall biosynthesis impacts defense against microbial pathogens. This picture has emerged from studies with biosynthetic inhibitors and analyses of mutants (often from genetic screens for altered pathogen resistance) and of plants in which cell wall components have been directly targeted in attempts to reduce recalcitrance.

Lignin

When flux into the lignin biosynthetic pathway is disrupted, plants can show compromised disease resistance. Early studies indicated that inhibition of the first phenylpropanoid biosynthetic enzyme PAL (**Figure 2*a***) by application of 2-aminoindan-2-phosphonic acid (AIP) or inhibition of the last enzyme in the monolignol pathway, cinnamyl alcohol dehydrogenase (CAD) (**Figure 2*a***), by application of [(2-hydroxyphenyl amino) sulfinyl] acetic acid 1,1-dimethyl ester (OH-PAS) increased disease susceptibility of *Arabidopsis* to *Peronospora parasitica* (80). In the case of CAD inhibition, it is likely that the effect was a result of reduced lignin content, but the results with PAL inhibitors might also reflect altered levels of antimicrobial phenylpropanoids or signaling molecules such as salicylic acid (SA), which can be derived from phenylalanine (80). Increased disease susceptibility of PAL-downregulated transgenic tobacco to *Cercospora nicotianae* was ascribed to reductions in chlorogenic acid (caffeoyl quinate) levels (76).

Defense responses have been studied in several *Arabidopsis* mutants in which the lignin pathway has been downregulated through either loss-of-function mutations in, or silencing of, monolignol pathway genes. The lack of activity of 4-coumaroyl shikimate 3′-hydroxylase (C3′H) (**Figure 2*a***) in the *ref8* (reduced epidermal fluorescence 8) mutant of *Arabidopsis* leads to defects in fungal defense (39). *CAD-C* and *CAD-D* function redundantly as components of basal resistance in *Arabidopsis* (120). The loss of function of both genes resulted in decreased resistance to both virulent and avirulent *Pseudomonas syringae* pv. *tomato*, although only *CAD-D* is strongly induced in response to these bacterial strains (120). Additionally, *CAD-C* and *CAD-D* may play indirect roles as possible regulators of SA-dependent defenses given that both SA production and the SA defense pathway were altered in the *cad-c cad-d* double mutant during compatible interactions (120). RNA-mediated transient gene silencing of the monolignol biosynthesis genes *PAL*, *COMT*, *CCoAOMT* (caffeoyl coenzyme A 3-*O*-methyltransferase) (**Figure 2*a***), and *CAD* compromised the defense of wheat against powdery mildew penetration to varying degrees, and cosilencing resulted in more severe defects than single gene silencing (8).

The alterations of lignin composition in response to infection may be important in determining the outcomes of some plant-pathogen interactions. For example, infection by, and reproduction of, root knot nematodes appears to be affected by S-lignin content. In *Arabidopsis* that highly over-accumulates S lignin as a result of overexpression of ferulate/coniferaldehyde 5-hydroxylase (F5H) under the promoter of the *C4H* (cinnamate 4-hydroxylase) gene, the development rate of the nematode *Meloidogyne incognita* was lower than in the wild-type control (136). Conversely, in

the tobacco *comt* mutant with reduced levels of S lignin, the life cycle of *M. incognita* progressed more quickly (136).

A single gene disruption in the lignin pathway can cause different effects on the susceptibility of a single species to different pathogens. For example, the *Arabidopsis comt1* mutant shows much lower levels of S lignin but similar overall lignin content to the wild type (47). Given that the transcriptional activation of *comt1* is responsive to infection with diverse pathogens, it is not surprising that this mutant displays weakened resistance to the bacterial pathogens *Xanthomonas campestris* pv. *campestris* and *P. syringae* pv. *tomato*, the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola*, and the biotrophic fungal pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) as well as to nematodes (102). The resistance of *Arabidopsis* to the necrotroph *A. brassicicola* requires the signal molecule jasmonic acid (JA) but not SA (128), whereas SA-dependent defense responses are involved in resistance to biotrophic fungal pathogens such as *Bgh* (143). Thus, mutation of *COMT1* compromises two different defense pathways against different types of pathogens. Unexpectedly, however, although the *COMT1* transcript level was increased after infection of *Arabidopsis* with the oomycete *Hyaloperonospora arabidopsidis*, sporulation levels were 40% to 50% lower on *comt1* mutants than on wild-type control plants; therefore, the *comt1* mutant is more resistant to this pathogen than is the wild type (102). The unexpected increase in resistance of the *comt1* mutant to *H. arabidopsidis* did not involve effects on SA- or JA-dependent defense pathways. Instead, overaccumulation of 5-hydroxyferuloyl malate (OH-FM), which is derived from monolignol precursors in the mutant, may account for the altered susceptibility through toxicity for the oomycete (102).

Disruption of monolignol biosynthesis can affect pathogen resistance through direct impacts on defense signaling pathways. For example, members of the Rac family of small GTPases play roles in regulating defense responses in many plant species (74, 84, 91, 93). As an example of this, rice OsRac1 regulates both NADPH oxidase activation for accumulation of reactive oxygen species (59) and the stability and activation of the MAP kinase AsMAP6, which is involved in pathogen defense (70). OsRac1 protein interacts with the monolignol pathway enzyme OsCCR1 in yeast, and OsRac1 can stimulate lignin synthesis in rice, presumably by regulating the activity of OsCCR1 during defense responses (60). In another example, NPR1 (nonexpressor of *PR* genes-1) is a central regulator of systemic acquired resistance (SAR) (29). Overexpression of a rice homolog of NPR1 (NH1) results in resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (22). A *CCR*-like gene, *SNL6*, is required for NH1-mediated resistance to *X. oryzae* pv. *oryzae* (6). This gene was identified by screening for suppressors of NH1-mediated lesion formation. *SNL6* is involved in lignin biosynthesis because the *snl6* mutant has reduced lignin content, overexpression of NH1 induces lignin biosynthesis, and the lignin pathway enzymes PAL and 4-coumarate Coenzyme A ligase (4CL) are highly coexpressed with *SNL6* (6). *SNL6* is required for NH1-mediated pathogenesis-related (PR) gene expression (6), but it also contributes to resistance in the absence of NH1 overexpression. *SNL6* may therefore have a dual function in defense against *X. oryzae* pv. *oryzae* through activation of *PR* genes and lignin biosynthesis (6).

Cellulose

Many mutants with impairment of the genes involved in cellulose biosynthesis [cellulose synthases (CESAs)] were first discovered through screening for mutants with altered disease resistance. In *Arabidopsis*, CESA4, CESA7, and CESA8 are required for cellulose production in the secondary cell wall (113, 119). In a screen for mutants that fail to develop disease symptoms caused by the necrotrophic fungus *Plectosphaerella cucumerina* or the bacterial pathogen *Ralstonia solanacearum*, CESA4/IRREGULAR XYLEM (IRX)5 and CESA8/IRX1 mutants were isolated

with enhanced disease resistance (52). CESA7 mutants also showed similar reduced susceptibility to both pathogens (52). Interestingly, resistance to *P. cucumerina* or *R. solanacearum* seems to be specifically activated by impairment of secondary as opposed to primary cell wall cellulose biosynthesis because other cell wall mutants, including those with primary cell wall cellulose biosynthesis defects that show enhanced disease resistance to biotrophic pathogens, have similar susceptibility to these two pathogens, as observed in wild-type plants (52). The reduced susceptibility was SA-, ethylene-, and JA-independent but required abscisic acid (ABA) (52).

Oligosaccharin: a sugar oligomer released from a plant cell wall polymer that possesses a signaling function

CESA3 encodes a cellulose synthase implicated in primary cell wall cellulose synthesis (113). The *CESA3* mutant *cev1* was isolated from a screen for constitutive activation of the JA defense response signaling pathway (30). The mutant has increased levels of JA and ethylene associated with constitutive activation of stress-related genes and enhanced resistance to powdery mildew (30).

The cyclic dipeptide phytotoxin thaxtomin A produced by *Streptomyces* species is an inhibitor of plant cellulose synthesis that causes aberrant cellulose synthase complex patterns in the plasma membrane (64). Treatment of *Arabidopsis* plants with thaxtomin A triggers ectopic lignification that is associated with expression of the defense-associated *CCR2* gene of monolignol biosynthesis and a suite of defense response genes, including markers of SA- (PR1, PR5) and JA- (VSP1, VSP2 and CHI-B) mediated pathways (9).

Mixed-linkage glucan (MLG) is, like cellulose, another primary cell wall glucose-derived polysaccharide. In rice, the loss-of-function mutant of the cellulose synthase-like *F6* (*CslF6*) gene, which is responsible for the biosynthesis of MLG, exhibited both decreased lesion size and reduced bacterial growth after infection with a virulent isolate of *X. oryzae* pv. *oryzae* (131). *PR* genes, other SA-responsive genes, and cell death–related marker genes were all significantly upregulated in non-inoculated *cslf6* mutant plants (131).

Hemicellulose and Pectin

Hemicellulose can negatively impact the accessibility of enzymes to cellulose, presumably through interactions with the cellulose microfibrils and cross-linkages with lignin polymers (53, 109). Hemicelluloses are structurally diverse, and the biological roles of their various decorations are still unclear, although the branches of the polymer influence its water solubility and interaction with cellulose and lignin.

Heterotrimeric G-proteins are involved in many different developmental processes and have been implicated in the regulation of cell wall architecture and hemicellulose (xylan) synthesis (27). In *Arabidopsis*, one gene encodes the Gβ subunit (*AGB1*) and three genes encode the Gγ subunits (*AGG1*, *AGG2*, and *AGG3*) of the heterotrimeric G-protein complex. The *Arabidopsis agg1 agg2* double mutant and *agb1* single mutant are more susceptible to the necrotrophic fungi *B. cinerea*, *P. cucumerina*, and *A. brassicicola* than are wild-type plants, but resistance to biotrophic fungi and bacterial pathogens appears to operate independently of heterotrimeric G-proteins (27, 121). The heterotrimeric G-protein mutants showed reduced levels of xylose, and their phenotypes were ascribed to changes in cell wall composition rather than to impacts on the SA, JA, ethylene, and ABA signaling pathways associated with resistance to necrotrophic pathogens (27).

Variation in pectin composition has also been associated with pathogen resistance. In a screen to recover *Arabidopsis* mutants with loss-of-susceptibility to the biotrophic pathogen powdery mildew, two of the so-called *pmr* (powdery mildew-resistant) mutants, *pmr5* and *pmr6*, showed altered pectin structures (132, 133). Their enhanced resistance was not dependent on SA, ethylene, or JA signal transduction pathways. It was hypothesized that novel pectin structures resulting from loss of function of PMR5 or PMR6 might be precursors for oligosaccharin signal molecules that elicit a defense response (133).

Many hemicellulosic or pectic plant cell wall polysaccharides are decorated with methyl, acetyl, or feruloyl groups linked to the sugar subunits. A loss-of-function mutant of one critical gene involved in polysaccharide *O*-acetylation, *REDUCED WALL ACETYLATION2* (*RWA2*), exhibited decreased levels of acetylated cell wall polymers (77). Polysaccharide *O*-acetylation inhibits hydrolase action and thus microorganisms would be expected to have easier access to the cell walls of the *rwa2* mutant. Interestingly, this mutant has enhanced tolerance toward the necrotrophic fungal pathogen *B. cinerea* and unaltered susceptibility to biotrophic fungal pathogens (77). Transgenically expressing hemicellulose- or pectin-specific fungal acetylesterases from *Aspergillus nidulans* significantly reduced cell wall polysaccharide acetylation in *Arabidopsis* and *Brachypodium* (100). The transgenic *Arabidopsis* and *Brachypodium* plants showed increased resistance to the fungal pathogens *B. cinerea* and *Bipolaris sorokiniana*, respectively, but the transgenic *Brachypodium* plants were not more resistant to the rice pathogen *X. oryzae*, and the transgenic *Arabidopsis* was not more resistant to *P. syringae* (100), which is consistent with the defense phenotype of the *rwa2* mutant with loss of function of the acetyltransferase (77).

Pectin provides a matrix that embeds the cellulose-hemicellulose network and is critical for tissue integrity and strength. Pectins are highly methyl-esterified but after secretion into the cell wall are de-esterified by pectin methylesterase (PME). De-esterification makes pectin more susceptible to degradation by pathogen-secreted pectic enzymes (14, 71, 73, 104), such as endopolygalacturonases (endoPGs) that can release elicitor active oligogalacturonides (36, 37). Tobacco and *Arabidopsis* plants overexpressing a fungal endoPG showed reduced homogalacturonan content (37) and were more resistant to the fungal pathogen *B. cinerea* (37). PME is regulated by specific proteinaceous inhibitors (PMEIs) (96). Transgenic plants with either overexpression of PMEIs or loss of function of endogenous PMEs show an enhanced degree of pectin methyl-esterification. *Arabidopsis AtPME3* is induced upon fungal infection and is necessary for initial colonization by necrotrophic fungi (104). The overexpression of *Actinidia chinensis* PMEI in wheat can reduce PME activity and therefore increase the degree of pectin methyl-esterification (134). Even though wheat, as a grass species, contains a low amount of pectin in the cell wall, the high degree of pectin methyl-esterification contributes significantly to resistance to fungal pathogens (134). Plant genomes also encode PMEIs to regulate pectin methyl-esterification, and plant PMEs are involved in many important physiological processes other than pathogen defense (55, 98). The *Arabidopsis* genome encodes two PMEIs, AtPMEI-1 and AtPMEI-2 (103). Constitutive expression of *AtPMEI-1* or *AtPMEI-2* in *Arabidopsis* increases pectin methyl-esterification and resistance to *B. cinerea* (73).

GENETIC ENGINEERING OF CELL WALL COMPONENTS TO IMPROVE FORAGE DIGESTIBILITY AND BIOFUEL PRODUCTION

Forage crops (usually grasses or legumes) are the primary feed utilized for ruminant animal production. However, lignification of plant tissues imposes a barrier to efficient cell wall digestion in the rumen and therefore negatively impacts forage digestibility (85). One study examining correlations between forage quality and disease resistance in an alfalfa improvement program showed that, out of 144 correlation coefficients computed between forage quality traits and resistances to five major alfalfa diseases, only six were significant but at low magnitude (38). It was therefore concluded that selection for forage quality is not expected to indirectly affect levels of disease resistance, although it may reduce vigor.

Genetic manipulation can generate significantly larger improvements in forage digestibility than can a single cycle of breeding. Genetic manipulation aimed at improving forage digestibility has primarily focused on downregulation of enzymes involved in the lignin biosynthetic pathway

Lignocellulose: a term that loosely defines the total cell wall material in a plant, which consists of cellulose, lignin, hemicelluloses, and pectins

(21, 49, 105, 110). In alfalfa, there is a strong negative linear relationship between lignin content of genetically modified plants and their dry matter forage digestibility or in vitro saccharification efficiency, whereas lignin composition, as reflected by S/G ratio, has little impact (105). For example, neither *Arabidopsis* nor alfalfa plants in which the ferulate/5-hydroxyconiferaldehyde 5-hydroxylase enzyme specific for S-lignin biosynthesis (**Figure 2*a***) has been downregulated, and which have drastically altered lignin composition but relatively normal lignin concentration, have altered cell wall digestibility (57, 105).

The same factors that negatively impact digestibility of forage crops present a major obstacle to economic feasibility of the lignocellulosic biofuel industry; essentially, the cellulosic and hemicellulosic polymers containing the sugars are not freely accessible to enzymes and/or microbes because of the inhibitory/masking effects of lignin and other features of cell wall structure (53). Studies on multiple plant species, but primarily on nonbiofuel crops such as transgenic alfalfa or mutant *Arabidopsis* with lignin biosynthesis that is disrupted at multiple independent steps, have revealed that saccharification efficiency is most strongly impacted by lignin concentration (20, 127), although other factors such as matrix polysaccharide content, overall galactose content, and fucose levels may also be important features of recalcitrance (112, 127). Several different strategies have been employed to target lignin for reducing recalcitrance in both model species and dedicated bioenergy crops. These include directly reducing lignin biosynthesis by disrupting the expression of the enzymes or regulatory transcription factors in the lignin biosynthesis pathway (20, 41, 78, 112, 140), engineering altered monomers that can block polymerization (138), or introducing novel lignin monomers into the polymer to render the lignin more susceptible to degradation during processing (33, 48). Although many of these approaches have been successful in terms of reducing cell wall recalcitrance, in many cases their impacts on plant performance and responses to biotic and abiotic stresses have yet to be fully evaluated.

Expression of microbial enzymes in planta to target cell wall polysaccharide structures that may contribute to recalcitrance, for example, through cell wall cross-linking, provides an alternative strategy for improvement of lignocellulosic bioenergy crops (99, 115). Recent studies have indicated that genetic modification of hemicellulose branch structure can increase fermentable sugar release from lignocellulose (23, 87). Constitutive targeting of an endo-xylanase from *Trichoderma reesei* to the vacuole or golgi in tall fescue resulted in reduced plant growth, whereas plants with the enzyme targeted to the apoplast, particularly when under control of a senescence-inducible promoter, exhibited increased levels of ferulate dimers, decreased levels of xylose, and increased levels of arabinose in their cell walls. However, high-level xylanase expression in the apoplast resulted in necrotic lesions on the leaves (18).

Pectin may also have negative impact on accessibility to cell wall–degrading enzymes during the process of biomass saccharification. For example, expression of a fungal polygalacturonase or an inhibitor of endogenous PME in *Arabidopsis* resulted in plants with a reduced content of demethyl-esterified homogalacturonan (HGA), and this was associated with increased efficiency of enzymatic saccharification (72). A fungal ferulic acid esterase from *Aspergillus niger* targeted to the vacuole of forage crops can remove ferulic acid from arabinoxylans and thereby make the cell walls more digestible (16, 17).

REDUCTION IN LIGNIN LEVEL DOES NOT NECESSARILY IMPAIR DEFENSE

Although reducing lignin content is currently considered to be the most efficient way to increase forage digestibility and biofuel production, this strategy is not viable if lower lignin content compromises defense systems (95). Fungal pathogens and insect pests present a major threat to the

successful establishment and sustainability of genetically engineered bioenergy (or forage) feedstocks (114), especially because these new lines will be, at least initially, in only one or at most a few different genetic backgrounds. It is now clear from a number of studies on naturally occurring mutants/variants, or transgenic plants in which lignin biosynthesis has been specifically targeted for the purpose of reducing cell wall recalcitrance, that reducing lignin content does not necessarily result in increased disease susceptibility. Much of this evidence has been reviewed recently (108).

Analysis of low-lignin brown midrib (*bmr*) sorghum mutants or *bm* maize mutants revealed, depending on the pathogen, either no impact on disease severity (sorghum/*Alternaria*) or enhanced resistance to some fungal pathogens (sorghum/*Fusarium* spp.) (42, 43, 107). Alfalfa plants exhibiting strong reduction in lignin levels and altered (H-rich) lignin composition through downregulation of the lignin pathway enzyme hydroxycinnamoyl Coenzyme A:shikimate hydroxycinnamoyl transferase (HCT) exhibited reduced stature (**Figure 2*b***) and deformed vascular tissues (**Figure 2*c***) but increased tolerance to *Colletotrichum trifolii* (**Figure 2*d***). These plants show enhanced transcript levels of a range of *PR* genes in the absence of pathogen inoculation, suggesting that the mechanism of resistance might involve cell wall–mediated defense signaling (45) (**Figure 2*e***) (see below).

Many low-lignin mutants or transgenics display impaired growth, with some plants, especially those downregulated in HCT, C3′H, or CCR, being quite stunted in appearance (68, 110, 142) (**Figure 2*b***). Most single mutants in CAD appear to have normal growth (possibly because of genetic redundancy due to the presence of a *CAD* gene family). However, the *Medicago truncatula cad1* mutant, although exhibiting a normal growth phenotype at 22°C, has a dwarf phenotype at 30°C, which is associated with expression of a set of pathogen defense genes only at the elevated temperature (141). This suggests that the defense response of some low-lignin plants might differ at different temperatures and not necessarily in the same way as that of the wild-type plant.

Although greenhouse and laboratory studies can indicate the potential for disease problems, there is no substitute for evaluation of plants under field conditions. Unfortunately, few studies of this type have been reported to date. Most involve plants with reduced lignin levels through targeting of one or another of the monolignol methylation enzymes COMT or CCoAOMT. Transgenic CCoAOMT downregulated low-lignin alfalfa plants have been evaluated in the field at multiple sites in the United States over multiple years; no evidence was obtained for yield losses that might reflect enhanced susceptibility to any natural pathogen (82). In contrast, the downregulation of COMT increased the rust susceptibility of transgenic perennial ryegrass grown in the field, although lignin modification by CCR downregulation did not (123). No instances of enhanced disease susceptibility in the field were reported for COMT-downregulated sugarcane or poplar (58, 97). Hybrid poplar lines expressing antisense COMT or CAD were as healthy as wild-type plants after four years' growth at two geographically different sites (50). Both rust (*Puccinia emaculata*) and *Bipolaris* species are potentially damaging diseases of field-grown switchgrass (125), and it is important to document the impacts of these pathogens in ongoing field trails of low-lignin switchgrass.

MECHANISMS UNDERLYING ACTIVATION OF DEFENSE PATHWAYS IN PLANTS WITH MODIFIED CELL WALLS

The fact that low-lignin transgenic plants, mutants or natural variants, do not always exhibit compromised pathogen resistance could be the result of a number of related or independent factors. One simple reason could be genetic redundancy; some lignin biosynthetic genes have family members that are not critical for lignin deposition during plant development but are responsive to pathogen attack. For example, as already discussed, *Arabidopsis* has more than one *CCR* gene,

and *CCR2* appears to be involved in pathogen resistance (67); when *CCR1* function is lost, *CCR2* expression is increased (83). Similarly, switchgrass possesses multiple *CCR* and *CCR*-like genes (32). The biochemical properties and expression pattern of switchgrass PvCCR1 are consistent with a role in lignification during development, whereas, as in *Arabidopsis*, CCR2 appears to be associated with plant defense (32). A second reason could be that blockage of lignin biosynthesis at certain steps can lead to accumulation of antimicrobial lignin precursors and/or their derivatives (83). Another possibility is that phenylpropanoid biosynthesis is wired into transcriptional regulatory networks that also control defense responses. Finally, reduced lignification might lead to changes in cell wall structure/integrity that favor release of oligosaccharins in the absence of a pathogen attack.

It has been known for a long time that monolignols, as well as the dimeric lignans that are derived from them, possess antimicrobial activity (5, 25), with lignans being commonly deposited as a pre-existing antimicrobial barrier in the heartwood of trees (116). Studies are now beginning to document the metabolomes of transgenic plants with altered lignin synthesis, and in some cases have reported accumulation of monolignols, lignans, and novel oligolignols (83, 86, 112, 122). In a detailed systems biology study of *Arabidopsis* mutants independently targeting ten genes of the monolignol pathway, the major metabolite changes reported involved phenylpropanoic acids, benzoic acid hexose esters (increased in most mutants), and dimers of coniferyl alcohol with ferulic acid (increased in some mutants and reduced in others) (130). COMT downregulated switchgrass plants accumulate a range of novel monolignol derivatives, such as iso-sinapyl alcohol and a number of yet to be identified lignans (122). In addition to potentially explaining altered disease resistance in these plants, some of these compounds might also act as fermentation inhibitors during bioethanol processing (122). Modification of lignin levels in switchgrass by overexpression of an MYB family transcriptional repressor (PvMYB4) led to reductions in the levels of several potential fermentation inhibitors, but levels of some lignans were increased (112); there were also changes in the ratios of wall-bound coumaric and ferulic acids that correlated with reduced recalcitrance. As a result of these and other structural changes in the cell walls, the PvMYB4 overexpressing plants produced as much as three times more ethanol per unit biomass than comparable controls (111, 112). The impacts of these manipulations on disease resistance have yet to be evaluated.

Transgenic alfalfa plants with lignin biosynthesis blocked at the steps catalyzed by HCT or C3′H (**Figure 2*a***) have increased levels of flavonoids and of the defense signal SA (45) (**Figure 2*e***). In fact, SA levels were inversely proportional to lignin levels in a population of transgenic alfalfa plants downregulated independently at different steps in the lignin pathway (44). The HCT and C3′H downregulated plants were of reduced stature (**Figure 2*b***) and strongly expressed a number of *PR* genes, which may account for their enhanced resistance to anthracnose (*Colletotrichum trifolii*) (45). Likewise, *Arabidopsis* plants downregulated in HCT exhibit a dwarf phenotype and have enhanced SA levels and *PR* gene expression (44). Crossing HCT-RNAi downregulated *Arabidopsis* plants with plants in which SA biosynthesis could not occur due to a block in its biosynthesis or in which SA was removed through expression of a bacterial salicylate hydroxylase gene led to a nearly complete restoration of growth and to a reduction in *PR* gene expression (44). It was hypothesized that alterations in cell wall integrity led to the release of cell wall components that triggered SA signaling and that SA was largely responsible for the alterations in both growth and defense in the transgenic plants (**Figure 2*e***). This model is, however, likely an oversimplification and has yet to be confirmed from studies in other plant species. In addition to increased SA levels, HCT downregulated alfalfa plants also exhibit changes in the levels of a number of growth regulators, including cytokinins and gibberellic acids (GAs), and show GA insensitivity (45) (**Figure 2*e***). Although SA may be orchestrating some of these effects, it is also possible that the hormonal changes arise by alternative mechanisms and may impact defense independently from SA.

Mediator is a multisubunit conserved transcriptional coregulatory complex that transduces information from *cis*-elements in gene promoters to the RNA polymerase II bound at the core promoter (62). Although its function in plants is complex and yet poorly understood, Mediator is involved in coordinating pathogen resistance (61). Recent studies suggest that Mediator is also critically involved in regulation of phenylpropanoid homeostasis in *Arabidopsis* (12), and dominant mutations in *REDUCED EPIDERMAL FLUORESCENCE 4* (*REF4*) in *Arabidopsis* result in reduced lignin deposition and dwarfing (12). Plants deficient in REF4 and the related protein RFR1 accumulate significant levels of compounds that arise from the coupling of monolignols with hydroxycinnamic acids (12). How these changes impact plant defense remain to be determined, but these studies suggest that there are complex and subtle transcriptional control mechanisms linking phenylpropanoid biosynthesis, lignification, growth, development, and likely pathogen resistance.

Whatever the ultimate cause of enhanced defense in some plant lines with modified cell wall structure might be, is there a common mechanism linking altered wall structure to defense gene activation? This perhaps seems unlikely when considering the fact that defense-related transcriptomic changes in low-lignin plants can differ not only between plants in which different lignin pathway genes are targeted (130) but also between plants of different species in which the same gene is targeted (26, 141).

Do targeted or off-target changes in cell wall structure following genetic modification somehow mimic the changes in wall structure seen during pathogen ingress and thereby lead to activation of endogenous signaling pathways? Cellulose microfibrils are not an attractive target of plant pathogens for degradation because of their tightly packed crystalline arrangement (28). However, pathogens secrete many degrading enzymes targeting hemicellulose and pectins (56), and the fragments released may be perceived by the plant as signals that assist in the orchestration of defense responses (135). Plant cell wall fragments that derive from pathogen infection have been called damage-associated molecular patterns (DAMPs) to distinguish them from pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), which are pathogen-derived elicitors (**Figure 1**). DAMPs are capable of triggering immune responses in plants (10). DAMPs were previously referred to as endogenous elicitors or, if derived from polysaccharides, as oligosaccharins (24).

The oligosaccharin hypothesis gained popularity in the late 1980s and early 1990s on the basis of the concept that plant cell walls do not simply serve a structural/protective function but also contain informational molecules that can regulate growth and development and/or act as signals that link pathogen recognition to subsequent defense responses (40). This field lost some traction in the 1990s, primarily because there were few ways to address the hypothesis beyond generating and testing various cell wall fractions in vitro. Attempts have recently been made to apply genetic approaches to understand oligosaccharin signaling and its potential dual role in defense and development (15, 126). The availability of a range of genetically modified, isogenic plant lines in which alterations of cell wall structure lead to expression of defense responses provides new genetic tools with which to test the hypothesis.

Although it has been assumed that there is little pectin present in secondary cell walls, this may simply be a result of its entrapment by lignin. Reducing lignin levels in secondary cell walls may potentially lead to diffusion of pectic fragments out of the wall, where they may be perceived by receptors in the plasma membrane (**Figure 1**) (15). Although there is as yet no direct genetic evidence to support a function for oligosaccharins in defense gene signaling in low-lignin plants, cell walls from HCT-downregulated alfalfa plants release more cold-water-soluble pectic material than do walls from wild-type plants, and the pectic material released from the low-lignin plants, but not from the wild type, can elicit induction of *PR* genes in alfalfa cell cultures (45). The exact structures of the elicitor-active materials remain to be determined.

Overexpression of a strawberry fruit–specific PME in fruits of wild strawberry resulted in constitutive activation of the defense response protein PR5, possibly as a result of increased SA levels, which are associated with increased resistance to *B. cinerea* (92). Size-exclusion chromatography and chemical degradation studies indicated that the transgenic plants contained smaller pectic oligomers with a reduced degree of methylation when compared with the pectic material from wild-type plants, and these molecules were highly active in eliciting *PR5* expression when injected into fruit; pectic material from wild-type plants was inactive, as were fully demethylated pectic oligomers from both wild-type and transgenic fruit (92). Recent advances in cell wall analysis, including whole cell wall NMR (79, 141) and glycome profiling (94), combined with the availability of new genetic systems (i.e., cell wall mutants), should greatly facilitate the reappraisal of the oligosaccharin hypothesis.

BALANCING CELL WALL MODIFICATION WITH DISEASE RESISTANCE AND AGRONOMIC PERFORMANCE FOR NEXT GENERATION BIOENERGY AND FORAGE CROPS

On the basis of the available literature, it appears that some (but not all) plants with strongly reduced recalcitrance might exhibit poor agronomic performance as a result of either defects in growth and development or altered susceptibility to pests or pathogens. It is also possible that plants that appear to perform satisfactorily in greenhouse or growth chamber studies might not perform well in the field. Several approaches could be taken to address and overcome these issues, with proof of concept already obtained for some.

As described above, hyperactivation of SA-mediated defense in plants often leads to dwarfing and growth abnormalities, and cell wall modification can turn on such defenses. However, a recessive mutant of *Arabidopsis*, *cdd1* (constitutive defense without defect in growth and development), exhibits constitutive activation of SA signaling pathways in the absence of pleiotropic growth effects (117), suggesting that it might prove possible to reverse engineer plants with cell wall modifications to restore growth without negatively impacting defense signaling. This hypothesis has yet to be demonstrated experimentally.

An alternative approach is to target lignin modification in such a way that it does not cause negative growth effects at the outset. Such an approach would maintain lignin in cell types in which its concentration was critical while reducing its level in other tissues or replacing it with increased levels of other cell wall polymers, such as cellulose. Theoretically, such an outcome could most easily be obtained by rewiring transcriptional control networks, and proof of concept for this approach was recently reported in *Arabidopsis* (137). The promoter of the early lignin biosynthetic gene *C4H* was replaced by the promoter of VND6, a transcription factor that confers vessel-specific expression. This was achieved by first obtaining a knockout mutant of the endogenous *C4H*. When the new copy of *C4H* is placed under expression of this transcription factor, the gene (and therefore lignification) is no longer expressed in the fibers. Along with this, polysaccharide deposition in the stems is enhanced by expressing a new copy of the fiber transcription factor NST1 under control of the promoter of the *IRREGULAR XYLEM 8* (*IRX8*) gene, which is itself upregulated by NST1. Because *C4H* is no longer under control of NST1, the fibers undergo deposition of more cell wall components under the autoregulatory NST1/IRX8 promoter feedback loop, but this material does not include lignin. Importantly, the growth of the engineered plants was normal (137), although impacts on plant defense that might occur as a result of misexpression of an early enzyme common to the biosynthesis of lignin and other phenylpropanoid-derived compounds remain to be determined.

An alternative approach to lignin engineering is to incorporate 4-*O*-methylated monolignols, which act to block polymerization (138). This was achieved by expressing an isoeugenol *O*-methyltransferase that naturally possessed 4-*O*-methylation activity for allyl/propenyl phenols but was engineered to have enhanced catalytic capacity for monolignols. The resulting *Arabidopsis* plants make significantly less lignin than the wild type and appear to exhibit normal growth and development; however, they accumulated an extended repertoire of phenolic esters as a result of the presence of the 4-*O*-methyl group (138). The impact of such changes on plant defense has yet to be reported.

The *Arabidopsis* SHINE clade of AP2 family transcription factors was first discovered as a result of their functions in controlling epidermal wax accumulation, which affects drought tolerance (2). Surprisingly, overexpression of AtSHN2 in rice led to a 45% reduction in lignin content accompanied by a 34% increase in cellulose, with no apparent negative impacts on plant strength or performance (4). Impacts on plant defense were not analyzed. It will be interesting to determine whether other cell wall polymers can substitute for the function of lignin as a constitutive defense barrier.

Alternative approaches seek to maintain the presence of lignin and alter its properties and structure by incorporation of novel lignin monomers. Several different strategies have been attempted, including truncation of the side chains of traditional monolignols through expression of a bacterial hydroxycinnamoyl CoA hydratase-lyase (33) and introduction of the building blocks into the polymer with more easily hydrolyzable linkages to render the lignin more susceptible to degradation during processing (48). These approaches remain to be evaluated in relation to how the plants will respond to microbial attack.

PERSPECTIVES

There is now abundant evidence that plant cell walls can be modified to reduce recalcitrance and thereby enhance digestibility of forage crops and processing for lignocellulosic-based biofuels. In the future, novel lignins will likely be engineered into plants as coproducts, and these, and other potentially useful polymers, will probably be targeted to the cell wall. However, because cell wall integrity is important in plant defense, there are potential conflicts between enhanced industrial value of a plant and compromised innate immunity. It will be important to be able to uncouple cell wall modification from its effect on pathogen defense. However, plant cell walls are highly complex in composition and structure, and there is variation in cell wall composition/structure within and among species. There is currently no easy way to determine exactly how differences in cell wall composition may impact the overall spectrum of disease resistance in a particular species. However, the availability of an increasing number of plant lines engineered in different ways for altered cell wall recalcitrance provides an excellent new resource for determining those features of plant cell wall structure that are limiting to pathogen ingress or that negatively impact the outcome of plant-pathogen interactions if modified.

SUMMARY POINTS

1. The structures of plant secondary cell walls have evolved to provide, among other things, physical protection against pathogen ingress. The composition of plant cell walls is dynamic and can change as an induced defense response.
2. The major impediment to the economic viability of lignocellulosic bioenergy crops is the recalcitrance of cell walls to hydrolysis to yield the component sugars of the major cellulosic and hemicellulosic polysaccharides.

3. Cell wall recalcitrance also limits the digestibility of forage crops for ruminant animals.
4. Recent studies have indicated that genetic engineering to alter the content and composition of plant cell wall polymers, particularly lignin, is a viable approach to reducing recalcitrance for improving forage and bioenergy crops, but this raises the question of whether such changes impact constitutive and inducible defenses against pathogens.
5. Alteration of plant cell wall structure by mutation or silencing of the genes involved in the biosynthesis of the major cell wall polymers can result in growth reductions and/or increased disease susceptibility or, paradoxically, enhanced disease resistance, depending on the gene targeted.
6. Some plants with reduced lignin levels constitutively express defense response genes in the absence of pathogen infection, suggesting that altered wall structure leads to release of oligosaccharide signals.
7. Strategies are being developed to uncouple reduced recalcitrance from negative impacts on plant growth; few of these studies to date have considered pathogen defense.
8. Field evaluations of reduced recalcitrance plants are critical but are in their infancy.

FUTURE ISSUES

1. Are the reduced growth and constitutive defense response phenotypes observed in some low-lignin plants linked, and can they be uncoupled from reduced recalcitrance?
2. Can cell wall composition be targeted in a highly tissue-specific manner to achieve reduced recalcitrance in the absence of negative impacts on growth and defense?
3. Can conventional lignin be replaced by a modified polymer that helps maintain cell wall integrity, prevents negative impacts on growth and defense, and facilitates biomass processing?
4. Do plant cell walls generate oligosaccharide signals that are specific for the types of pathogen or pest that releases them, and are the same molecules released more or less easily from plant cell walls with genetically altered composition?

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