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Modifying lignin to improve bioenergy feedstocks: strengthening the barrier against pathogens?†

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INTRODUCTION

In the U.S. and around the world, there are increasing efforts to develop and utilize alternatives to fossil fuels to meet our energy needs, thereby reducing carbon dioxide emissions that potentially impact global warming. Currently, corn grain and sugarcane juice are being converted into ethanol for blending in gasoline. Research efforts have been directed toward developing means to convert plant biomass from a range of sources into liquid fuels for the transportation sector. Cellulosic biofuels rely on chemically and biochemically breaking down cell wall polysaccharides (cellulose and hemicellulose) into their sugar monomers, and converting the sugar into fuels. A third component of cell walls is the phenolic polymer lignin, which structurally fortifies the cell walls making them rigid and resistant to microbial degradation. Lignin content has been shown to negatively impact cellulosic bioenergy conversion via saccharification and fermentation to ethanol (Chen and Dixon, 2007; Dixon, 2007), which has made reducing lignin and altering lignin composition a major target to improve plants for cellulosic bioenergy. Conversely, increasing the lignin content of herbaceous feedstocks may benefit conversion of biomass to syngas and bio-oil biofuel via pyrolysis. In either case, efforts to manipulate lignin content and composition have primarily focused on the 10 steps of the monolignol pathway (Figure 1), in which lignin monomers are synthesized from the amino acid phenylalanine, then oxidatively polymerized into hydroxyphenol-(H-), guaiacyl-(G-), or sinapyl-(S-) lignin. Lignin serves the critical function of reinforcing vascular elements for water transport under negative pressure; in severely lignin deficient plants, vascular collapse has been observed (Piqureral et al., 1998; Jones et al., 2001; Ruel et al., 2009). Thus, there is a lower limit for lignin manipulation. In addition to its role in fortifying cell walls, lignin deposition has long been implicated as an important defense mechanism against pests and pathogens (Vance et al., 1980). A critical question for bioenergy feedstock development is whether manipulating lignin content and composition will be detrimental to plant defenses against pathogens. Herein, we examine this question and the cause for concern in manipulating lignin, based on current published literature.

ROLE OF LIGNIN IN PLANT DEFENSE

There is a strong case for the involvement of lignin in plant defense. Lignin provides a physical barrier against initial ingress (Burenden et al., 1990; Boneillo et al., 2003), and in a wide range of plant species lignin or lignin-like phenolic polymers are induced and rapidly deposited in cell walls in response to both biotic and abiotic stresses, which may limit further growth and/or confine invading pathogens (Siegrist et al., 1994; Lange et al., 1995; Baayen et al., 1997;...)
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FIGURE 1 | A model for monolignol pathway.

In phenylpropanoid metabolism, there are 10 enzymatic steps (green) leading to hydroxycinnamyl alcohols which are polymerized into lignin, namely: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), hydroxycinnamoyl CoA:shikimate transferase (HCT), p-coumarate 3-hydroxylase (C3'H), caffeoyl CoA O-methyltransferase (CCoAOMT), cinnamyl CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid O-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD).

1996; Smit and Duberry, 1997; Bonello et al., 2003; Hudgins et al., 2004; Wuyts et al., 2006; Menden et al., 2007). “Defense” lignin may prevent further ingress or diffusion of pathogen-produced toxins (Carver et al., 1994; Duschnicky et al., 1998). However, defense lignin deposition is often only monitored microscopically as cell wall autofluorescence or via histochemical staining techniques (Haegi et al., 2008; Eynck et al., 2009). Defense lignin was often shown to have elevated levels of H-subunits as compared to structural lignin in the cases analyzed (Ride, 1975; Hammerschmidt et al., 1985; Doster and Bostock, 1988; Robertson and Svalheim, 1990; Lange et al., 1995). The phenylpropanoid pathway leads to the synthesis of numerous other phenolic compounds besides monolignols, including phenolic phytoalexins, stilbenes, coumarins, and flavonoids (Lo and Nicholson, 1998; Yu et al., 2000, 2005; Dixon et al., 2002). A number of these compounds have also been implicated in plant defense (Wierenga et al., 1996; Dicko et al., 2005; Lozovaya et al., 2007). For example, the defense signaling hormone salicylic acid (SA) might also be derived from the phenylpropanoid pathway in some plants (Ruohola and Jalkunen-Titto, 2003; Pan et al., 2006). Moreover, abiotic or biotic stresses including pathogens have been shown to induce the expression of genes encoding monolignol biosynthetic enzymes in many plant species (Kliebenstein et al., 2002; Truman et al., 2006; Olsen et al., 2008; Zhao et al., 2009). Likewise, the protein levels and enzymatic activities corresponding to these genes were also shown to be elevated under these stresses in a number of plant species (Mitchell et al., 1999). Together these observations indicate that lignin deposition is part of a generalized resistance response to biotic stresses (Nicholson and Hammerschmidt, 1992). Thus, it remains to be determined whether bioenergy crops that are impaired or altered in their ability to synthesize lignin will also be impaired in their ability to induce these defense responses upon pathogen attack. Recent research has suggested that impairing lignin biosynthesis does not lessen resistance to some pathogens (Delgado et al., 2002; Funnell and Peder sen, 2006; Peltier et al., 2009; Quentin et al., 2009; Funnell-Harris et al., 2010). Because very little has been published on the effects of lignin modification on plant-pathogen interactions in bioenergy feedstocks with the exception of maize (Zea mays) and sorghum (Sorghum bicolor), we have also included a review of the literature on effects of impairing steps in the monolignol pathway to pathogen responses in other plant species.

However, several pathogens have been isolated and identified that pose potential threats to some of the perennial grass species being considered as herbaceous bioenergy feedstocks including switchgrass (Panicum virgatum), napiergrass (Pennisetum purpureum), sugarcane/energycane (complex hybrid Saccharum spp.), and miscanthus (Miscanthus × giganteus). Pathogens may pose a greater threat to perennial grasses as compared to annual row crops such as maize and sorghum, because production relies on establishment and harvest across multiple years before replanting, and the continual presence of the plants in the field provides refuge for the pathogens. Another factor that could impact plant–pathogen interactions is the level of genetic diversity within the field setting. Switchgrass varieties are maintained as an outcrossing population (Martinez-Reyna and Vogel, 2002; Nagyowa-Rao et al., 2012), hence maintain level genetic diversity. In contrast, the clonally propagated miscanthus is genetically identical (Lewandowski et al., 2000). Fungal leaf rusts caused by Puccinia
Anthracnose, a foliar blight caused by
4 PAL genes. T-DNA insertion
Arabidopsis
2003). A fungal smut has been identified on
us (Chatani et al., 1991; Turina et al., 1998; Lamptey et al.,
which have been documented in switchgrass and miscan-
gal pathogens pose a serious threat to these bioenergy crops.
Overall, these studies indicated that similar to row crops, fun-
resulted in increased susceptibility to TMV although resistance to
(NahG), which degrades SA, in concert with P AL over-expression,
microbe freestocks, which have been documented in switchgrass and miscan-
thus (Chatani et al., 1991; Turina et al., 1998; Lamptey et al.,

PHENYLALANINE AMMONIA LIASE
Phenylalanine ammonia lyase (PAL) is the first committed step
in monolignol biosynthesis and the phenylpropanoid pathway.
Altering the expression of this central gene has been shown to
impact plant–pathogen interactions in model systems. In tobacco
(Nicotiana tabacum), antisense suppression of this gene led to
increased susceptibility to the fungal pathogen Cercospora nicotiana,
the causal agent of frogeye disease (Maher et al., 1994; Shadle et al., 2003).
Tobacco plants over-expressing this gene had reduced susceptibility to Cercospora nicotiana, but resis-
tance to tobacco mosaic virus (TMV) was unchanged (Shadle et al., 2003). Over-expression of PAL in tobacco resulted in sig-
ificantly increased levels of the defense signal compound
SA and the defense related compound chlorogenic acid upon induction
(Howles et al., 1996; Felton et al., 1999). Further-
more, over-expressing the bacterial salicilate hydroxylase gene
(NahG), which degrades SA, in concert with PAL over-expression, increased susceptibility to TMV although resistance to Cercospora nicotiana was unaffected (Shadle et al., 2003). These results indicated that TMV resistance required SA but not chlorogenic acid, while increased resistance to Cercospora nicotiana only required elevated levels of chlorogenic acid and not SA (Sha-
dle et al., 2003). Conversely, PAL over-expression in tobacco resulted in increased susceptibility to the insect Heliothis virescens, and a PAL-suppressed line had increased resistance, which was attributed to the antagonistic relationship between SA signal-
ing and jasmonic acid (JA) signaling (Felton et al., 1999).
The Arabidopsis genome contains four PAL genes. T-DNA insertion
mutants were isolated for all four genes, and these mutants were crossed to create double, triple, and quadruple pal mutants (Huang et al., 2010). The pal1/2/3/4 quadruple mutant showed increased susceptibility to the bacterial pathogen Pseudomonas syringae relative to WT, and pal1/2 also had increased suscepti-
tibility to this pathogen relative to WT and intermediate to
pal1/2/3/4 (Huang et al., 2010). SA, lignin and anthocyanin related pigment levels were significantly reduced in pal1/2/3/4 plants (Huang et al., 2010). However, these changes in susceptibility to pathogens cannot be directly attributed to changes in lignin
content or composition, because PAL is involved in the synthe-
sis of the full range of phenolic compounds, some of which have been implicated in defense, like chlorogenic acid and flavonoid
compounds.

HYDROXYCINNAMOYL CoA SHIKIMATE TRANSFERASE
In Arabidopsis (Arabidopsis thaliana) and alfalfa (Medicago sativa), antisense/RNAi suppression of hydroxycinnamoyl CoA:shikimate transferase (HCT) one of the initial steps in monolignol biosyn-
thesis, illustrates the potential of genetic/taxonomic alterations to
this pathway to constitutively activate defenses (Gallego-Giraldo et al., 2011a,b). In both plant species, antisense/RNAi suppression of HCT resulted in significant reductions in lignin content and stunted plants relative to WT (Shadle et al., 2007; Li et al.,
2010). In alfalfa, these plants showed increased resistance to the fungal pathogen Colletotrichum trifolii (Gallego-Giraldo et al., 2011b). In the absence of a pathogen, SA levels were highly ele-
vated relative to WT in both species and several defense related genes were also highly induced relative to WT in alfalfa (Gallego-
Giraldo et al., 2011a,b). In Arabidopsis, growth was partially restored in NahG HCT-RNAi and SA induction deficient2-2 (sid2-
2) HCT-RNAi; SID2 encodes an isochorismate synthase required for isochorismate-dependent SA synthesis. These results indicate that the stunted growth phenotype is due to elevated SA, occur-
ring through an isochorismate-dependent pathway, rather than from excess phenylalanine intermediates leading to the synthesis of SA in HCT-RNAi plants. Elevated levels of cold-water extractable pectin were correlated to elevated SA levels in trans-
genic alfalfa plants, which were RNAi-suppressed for six different genes (stems) in the monolignol pathway (Gallego-Giraldo et al.,
2011a). Highest levels of SA and cold-water extractable pectin were observed in HCT-suppressed lines relative to WT or the other five monolignol biosynthetic gene-suppressed lines (Gallego-
Giraldo et al., 2011a). Pectic oligosaccharides have been implicated as defense signals in other systems (Davväl and Albersheim, 1984; Rocso et al., 1993), and are the potential trigger for the defense responses observed in HCT lines. Thus, the effects observed in the HCT-suppressed lines could potentially result from changes in
cell wall structure, the first line of defense for the plant, rather than directly resulting from an alteration in phenylpropanoid
metabolism.

CAFFEIC O-METHYLTRANSFERASE
In Arabidopsis and tobacco, antisense/RNAi suppression of caffeic
O-methyltransferase (COMT), the penultimate step in monolign-
ol biosynthesis, was reported to increase resistance to pathogens or to have no effect on interaction with pathogens. In Arabidop-
sis, comt mutants show enhanced resistance to the oomycete pathogen Hyaloperonospora arabidopsidis, which is the causal agent of downy mildew (Quentin et al., 2009). There were sig-
ificantly fewer asexual spores on comt plants relative to WT, because sexual sporulation was increased in comt plants, resulting in attenuated mycelium growth (Quentin et al., 2009). Exposing the pathogen to the phenolic compound 2-O-5-hydroxyferuloyl-
malate, which is present in comt and absent in WT plants, promoted sexual reproduction (Quentin et al., 2009). However, comt plants showed increased susceptibility relative to WT to the
bacterial pathogens Xanthomonas campestris pv. campestris and Pseudomonas syringae and a less virulent strain (T4) of the fungal pathogen Botrytis cinerea (Quentin et al., 2009). In tobacco, COMT antisense lines were resistant to Agrobacterium tumefaciens infection, and had reduced tumor area and mass relative to WT (Maury et al., 2010). Bacterial virulence (vir) gene induction was reduced in the COMT-suppressed line likely due to the highly reduced level of the phenolic elicitor of Agrobacterium actinorhyzine (Mauery et al., 2010). Actinorhizin is probably derived from Cinnamyl Alcohol Dehydrogenase (CAD) gene, the last step in monolignol biosynthesis. This result might have implications for bioenergy feedstock improvement.

**CINNAMYL ALCOHOL DEHYDROGENASE**

In flax (Linum usitatissimum L.), RNAi suppression of the cinnamyl alcohol dehydrogenase (CAD) gene, the last step in monolignol biosynthesis, increased susceptibility to the pathogenic fungus Fusarium oxysporum. A seedling assay showed the percent of infected seedlings was twofold higher in two CAD RNAi lines relative to WT (Wooßel-Kloczkowski et al., 2007). In Arabidopsis, the cad-c and cad-d double mutants, which were shown to be required for monolignol biosynthesis (Kim et al., 2004; Silhout et al., 2005), showed increased susceptibility to both a virulent and an avirulent strain of the bacterial pathogen Pseudomonas syringae pv. tomato (Pst DC3000, virulent; DC3000avrPphB, avirulent) relative to WT based on bacterial growth following inoculation (Tischler et al., 2010). Together, these results suggest that CAD deficiency may increase the susceptibility of plants to a range of pathogens. This result might have implications for bioenergy feedstocks, because CAD suppression is often targeted to reduce lignin content.

**OTHER STEPS IN MONOLIGNOL SYNTHESIS**

In Arabidopsis, the ferulate 5-hydroxylase 1 (fah1) mutant, which encodes the ferulic acid 5-hydroxylase (F5H), last hydroxylase in monolignol synthesis, showed increased susceptibility to the fungal pathogen Sclerotinia sclerotiorum relative to WT in leaf assays (Huang et al., 2009). In diploid wheat (Triticum monococcum L.), five genes in monolignol biosynthesis were transiently silenced using particle bombardment of an RNAi vector containing PAL, cinnamoyl-CoA O-methyltransferase (CCoAMT), 4CL, COMT, or CAD genes (Bhujain et al., 2009). The bombardated leaves were inoculated with the powdery mildew fungal pathogens Blumeria graminis f. sp. tritici (host-specific) and Blumeria graminis f. sp. hordei (non-host). The silencing of all five genes individually and in pairs increased the susceptibility to both pathogens relative to the control bombardered with the empty RNAi vector, as determined by penetration efficiency of the fungus (Bhujain et al., 2009). However, it is unclear whether this transient approach to gene silencing is relevant to the stable approaches used to impair genes within this pathway for bioenergy feedstock improvement.

**BIODIVERSITY FEEDSTOCKS**

There has been very little published on plant pathogen interactions in bioenergy feedstocks with modified lignin content and composition. In hybrid poplar (Populus tremula x Populus alba), it has been reported that no increased disease incidence were observed in antisense COMT or CAD lines relative to WT (Halpin et al., 2007). The one exception where the effects of lignin modification on plant pathogen interactions has been examined are the brown midrib (bmr/bm) mutants of sorghum and maize (Zea mays), which have long been known to have reduced lignin content (Jorgenson, 1931; Porter et al., 1978). There are at least five Bmr loci identified in maize (Chen et al., 2012) and at least seven Bmr loci in sorghum (Pedersen et al. unpublished). Three Bmr loci have been cloned and characterized in sorghum. Bmr2, Bmr6, and Bmr12 all encode enzymes in monolignol biosynthesis: a 4-coumarate coenzyme A ligase (4CL), a CAD, and a COMT, respectively (Bout and Vermerris, 2001; Saballos et al., 2009; 2012; Sattler et al., 2009). In maize, the Bmr locus encodes a CAD protein (Vignoles et al., 1999) that is orthologous to Bmr12, and the Bmr1 locus encodes a CAD protein that is orthologous to Bmr6 (Halpin et al., 1998; Chen et al., 2012). Lignin deposition and the induction of phenylpropanoid-related genes during pathogen attack (described above) led to the assumption that brown midrib plants are inherently more disease susceptible when challenged. However, studies examining both grain and stalk fungal pathogens, which are the most prevalent and economically significant sorghum pathogens (Chandrabhak and Satyanarayana, 2006), have in general indicated the contrary.

Fungal infection of bm6/bm6 grain may not appear to be relevant to bioenergy, however, fungal infection of grain can impair seed germination (Ran et al., 1999; Proem et al., 2003), which is critical for all cropping systems. Under field conditions without inoculation, maize bm3 grain showed significantly increased colonization by members of the Gibberella fujikuroi fungal species complex as compared to WT grain (Nicholson et al., 1976). In contrast, studies using uninoculated field-grown sorghum showed that bm6 and bm12 grain had the same level of colonization or significantly reduced fungal colonization relative to WT, which included the sorghum pathogen P. sajor-caju, a G. fujikuroi species complex member (Funnell and Pedersen, 2006; Funnell-Harris et al., 2010). Other Fusarium spp. colonized both bm6 and bm12 grain at similar levels or significantly reduced colonization relative to WT (Funnell and Pedersen, 2006; Funnell-Harris et al., 2010). In particular, two species that commonly infected WT grain were significantly reduced or absent in bm12 grain, F. proliferatum and a member of the F. incarnatum-E. equiseti species complex (O’Donnell et al., 2007), respectively (Funnell-Harris et al., 2010). Taken together, these results indicated that impairing CAD or COMT activity in sorghum did not increase susceptibility to these Fusarium spp., and bm12 grain restricted or excluded colonization of two species. These results contradict the single early report from maize where bm3 grain, which is also COMT-deficient, showed increased colonization by the G. fujikuroi species complex (Nicholson et al., 1976).
Studies examining the susceptibility of maize and sorghum bm/bmr mutants to stalk rot pathogens, which impact biomass quality and can contribute to lodging, also showed no change in resistance or increased resistance relative to WT, similar to the grain studies. *F. thaapsinum* was inoculated in the peduncles of *bm1*, *bm12*, and WT from six near-isogenic backgrounds and disease severity was determined by the length of the purple disease lesion resulting from the fungal infection. Lesion lengths were significantly shorter than corresponding WT backgrounds for many *bm1* and *bm12* lines, and lesion lengths were significantly shorter than WT for one or both *bm* lines across four different genetic backgrounds (Funnell and Pedersen, 2006; Funnell-Harris et al., 2010). There were no cases where the lesion length was significantly greater in a *bm* line relative to the corresponding WT line (Funnell and Pedersen, 2006; Funnell-Harris et al., 2010). Peduncule inoculations of *bm6*, *bm12*, and wild-type lines with four *Fusarium* species and *Alternaria alternata* consistently resulted in decreased lesion lengths on one or both *bm* mutants relative to WT for the following pathogens; *F. thaapsinum*, *F. verticillioides* and *Alternaria alternata* (Funnell-Harris et al., 2010). Overall, these results consistently indicated that *bm6* and *bm12* were not more susceptible to these pathogens than WT, and in some cases the two *bm* mutants appeared to be more resistant to specific pathogens relative to WT. However, fungal viability was assessed within the lesions and outside the lesions, and fungal growth was detected within and outside borders of lesions from *bm12* inoculated peduncles (Funnell-Harris et al., 2010). This result suggests that fungal growth is greater in healthy-appearing tissues outside the necrotic, discolored tissue defined as the “lesion” in *bm12* plants, although these lesions were similar in size or significantly shorter than WT in *bm12* peduncles. Nevertheless, CAD or COMT deficiency in sorghum does not appear to significantly increase susceptibility of plants to these stalk rot pathogens.

A study using inoculations of another fungal stock pathogen *Macrophomina phaseolina*, which causes charcoal rot, also demonstrated *brown midrib* mutants were not more susceptible to this pathogen. *bm* mutants from sorghum (*bm2*, *bm6*, *bm7*, *bm12*, *bm26*, and *bm28*; three loci, *bm28* is allelic to *bm6*, and *bm7* and *bm26* are allelic to *bm12*, Saballos et al., 2008) and maize (*bm2*, *bm3*, *bm4*, and *bm6*; four loci) were inoculated with *Macrophomina phaseolina* and lesion lengths were compared to corresponding WT lines (Teso and Ejeta, 2011). Lesion lengths were not significantly different between *bm/bmr* mutants and the corresponding WT backgrounds for both maize and sorghum (Teso and Ejeta, 2011). Stalk strength as determined using ring penetration resistance was significantly reduced in maize *bm* mutants relative to WT (Teso and Ejeta, 2011). Interestingly, reduced mechanical stalk strength did not appear to increase susceptibility (Teso and Ejeta, 2011). However, all studies relied on artificial inoculation to ensure a consistent disease response. If decreased ring penetration resistance (stalk strength) increases the ability of the fungi to initially enter and penetrate the stalk, then results from these studies may be misleading. All the *bm/bmr* mutants examined resulted in similar susceptibility to the charcoal rot pathogen, even though at least three different steps in monolignol biosynthesis were impaired by the corresponding *bm* mutation; 4CL (*bm2*), COMT (*bm3/bm12*), and CAD (*bm1/bm6*). The general trend from these studies indicate that maize and sorghum *brown midrib* mutants are not more susceptible to stalk rot pathogens, and in some cases show increased generalized resistance to specific pathogens.

There are several explanations for the instances of increased generalized resistance observed in the *brown midrib* mutants. Although the ability of these *bm* plants to synthesize structural lignins is decreased and/or altered, there is no evidence *bm* plants are impaired in their ability to synthesize “defense” lignin in response to pathogen attack, and the response might even be enhanced. Another explanation is that blocking a step in the lignin biosynthetic pathway would cause accumulation of lignin precursors and other phenolic compounds, because additional substrates would be available for their synthesis. Indeed it has been shown that some of these precursors inhibit the growth of pathogenic fungi or inhibit production of virulence factors (Dowd et al., 1997; Hua et al., 1999; McKeen et al., 1999; Beerkum et al., 2003). For example, accumulation of ferulic acid, *p*-coumaric acid, and sinapic acid has been correlated with resistance to *Fusarium* spp. (McKeen et al., 1999; Strainidou et al., 2002). We have observed increased soluble phenolic compounds in *bm6* and *bm12* plants relative to WT (Palmer et al., 2008). Alternatively, perturbing the synthesis of lignin, a component of the cell wall which is the first line of defense against pathogens, could trigger generalized cell wall based defense responses similar to HCT-RNAi lines in *Arabidopsis* and alfalfa (Gallego-Giraldo et al., 2011a). A review focused on the broader role of the cell wall in plant defense was previously published (Underwood, 2012), which documents the significance of the plant cell wall in responses to a wide range of pathogens.

**PROSPECTIVE**

These studies from a variety of plants indicate that reducing lignin content and altering its composition will not inevitably increase the susceptibility of bioenergy feedstocks to pathogens. There were not any clear trends that indicate that impairing a specific step in monolignol biosynthesis would affect plant susceptibility. In fact, studies from sorghum and maize indicate that impairing CAD or COMT activity in these lignin-modified plants showed more resistance to specific fungal pathogens, albeit these plants are not as resistant to the pathogen as resistant plant germplasm used in breeding efforts. In bioenergy feedstock species, modifications to monolignol biosynthesis will need to be evaluated on a case by case basis to determine the impact of pathogen susceptibility.

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