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 chemical and biologically 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 cellulose bioenergy conversion via saccharification and fermentation to ethanol (Chen and Dixon, 2007; Dinn et al., 2009), 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 hydroxyphenyl- (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 (Piquemal et al., 1998; Jones et al., 2001; Ruel et al., 2009). Thus, there is a lower limit for lignin manipulation. In addition to its roles 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 (Borrelli et al., 1990; Bonello 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.,

Lignin is a ubiquitous polymer present in cell walls of all vascular plants, where it rigidifies and strengthens the cell wall structure through covalent cross-linkages to cell wall polysaccharides. The presence of lignin makes the cell wall recalcitrant to conversion into fermentable sugars for bioenergy uses. Therefore, reducing lignin content and modifying its linkages have become major targets for bioenergy feedstock development through either biotechnology or traditional plant breeding. In addition, lignin synthesis has long been implicated as an important plant defense mechanism against pathogens, because lignin synthesis is often induced at the site of pathogen attack. This article explores the impact of lignin modifications on the susceptibility of a range of plant species to their associated pathogens, and the implications for development of feedstocks for the second-generation biofuels industry. Surprisingly, there are some instances where plants modified in lignin synthesis may display increased resistance to associated pathogens, which is explored in this article.

Keywords: plant pathogens, lignin, brown midrib, monolignol pathway, CAD, COMT
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), \( \text{p-coumarate} \) 3-hydroxylase (C3'H), cinnamoyl-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 Dubery, 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 (Wieder gang 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 (Ruoshola and Jukunen-Tiitto, 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; Olesen 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 Ham merschmidt, 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 Pedersen, 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 (Martínez-Reyna and Vogel, 2002; Nageswara-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
Altering the expression of this central gene has been shown to increase susceptibility to TMV although resistance to 
*(Pseudomonas syringae)*, which degrades SA, in concert with *P AL* over-expression, was unaffected (Shadle et al., 2003). These results indicated that TMV resistance required SA but not chlorogenic acid, while increased resistance to the fungal pathogen *Hyaloperonospora arabidopsidis* (Quentin et al., 2009). Pectic oligosaccharides have been implicated as defense signals in other systems (Darvill and Albersheim, 1984; Roso 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 monolignol biosynthesis, was reported to increase resistance to pathogens or to have no effect on interaction with pathogens. In *Arabidopsis*, *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 significantly 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-S-hydroxyferuloyl-L-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 (Quarin 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 acetylsyringone (Maury et al., 2010). Acetylsyringone is probably derived from Coenzyme A dependent β-oxidation of hydroxycinnamoyl-CoA intermediates of monolignol biosynthesis (Blouot et al., 2002; Negrel and Javelle, 2010). In Arabidopsis and tobacco, the alteration to phenylpropanoid metabolism by reducing COMT activity appears to directly result in increased resistance to two of the pathogens tested, downy mildew and Agrobacterium, respectively. However, the same Arabidopsis plants showed increased susceptibility to two bacterial pathogens and a less virulent strain of Botrytis cinerea.

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 F. oxysporum. A seedling assay showed the percent of infected seedlings was twofold higher in two CAD RNAi lines relative to WT (Woolbel-Kowatkowska 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; Silbrowitz et al., 2005), showed increased susceptibility to both a virulent and an avirulent strain of the bacterial pathogen Pseudomonas syringae pv. tomato (Pat;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 ferulic acid 3-hydroxylase 1 (fah1) mutant, which encodes the ferulic acid 3-hydroxylase (F3H), 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, caffeoyl-CoA O-methyltransferase (CCoAMT), F3H, CAD, or COMT genes (Bhuiyan et al., 2009). The bombarded 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 susceptibility to both pathogens relative to the control bombarded with the empty RNAi vector, as determined by penetration efficiency of the fungus (Bhuiyan 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.

BIOENERGY 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 × 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 (Jogensen, 1931; Porter et al., 1978). There are at least five Bm loci identified in maize (Chen et al., 2012) and at least seven Bm 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 (O’Donnell et al., 1999, Proem et al., 2003), which is critical for all cropping systems. Under field conditions without inoculation, maize bmr3 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 bmr6 and bmr12 grain had the same level of colonization or significantly reduced fungal colonization relative to WT, which included the sorghum pathogen P. hamiltonii, a G. fujikuroi species complex member (Funnell and Pedersen, 2006; Funnell-Harris et al., 2010). Other Fusarium spp. colonized both bmr6 and bmr12 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 bmr12 grain, F. proliferatum and a member of the F. incarnatum-F. 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 bmr12 grain restricted or excluded colonization of two species. These results contradict the single early report from maize where bmr3 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. \( F.\) *thapsinum* was inoculated in the peduncles of \( bm/bm \) 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 both maize and sorghum \( F.\) *thapsinum* (*Tesso* and *Ejeta*, 2011). Stalk strength as determined with \( M.\) *phaseolina* mutants and the corresponding WT backgrounds for both maize and sorghum \( bm/bmr \) mutants were 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 \( bm/bmr \) mutants. Although the ability of these \( bm/bm \) plants to synthesize structural lignins is decreased and/or altered, there is no evidence that \( bm/bm \) plants are impaired in their ability to synthesize “defense” lignin in response to pathogen attack, and the response might even be increased. 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; *McKeren* et al., 1999; *Beekrum* et al., 2003). For example, accumulation of ferulic acid, \( p\)-coumaric acid, and sinapic acid has been correlated with resistance to *Fusarium* spp. (*McKeren* et al., 1999; *Strainidou* et al., 2002). We have observed increased soluble phenolic compounds in \( bm/bm \) and \( WT \) 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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Lignin modification and pathogens

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