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RESEARCH

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Synergetic effect of fungal pretreatment and lignin modification on delignification and saccharification: a case study of a natural lignin mutant in mulberry

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Abstract

Background Fungal pretreatment for partial separation of lignocellulosic components may reduce lignocellulose recalcitrance during the production of biofuels and biochemicals. Quantitative and qualitative modification of plant lignin through genetic engineering or traditional breeding may also reduce the recalcitrance. This study was conducted to examine the effects of combining these two approaches using three white rot fungi and mulberry wood with an altered lignin structure.

Results Mulberry wood prepared from homozygotes or heterozygotes with a loss-of-function in the cinnamyl alcohol dehydrogenase gene (*CAD*) was pretreated with three fungal species. Both heterozygous (*CAD*/*cad*) and homozygous (*cad*/*cad*, null mutant) mulberry plants were derived from the same parents via backcrossing between Sekizaisou (*cad*/*cad*, seed parent), a natural lignin mutant, and its F1 progeny (*CAD*/*cad*, pollen parent). Homozygote wood and the isolated lignin exhibited an abnormal color. Lignin in homozygotes without fungal pretreatment exhibited a lower syringyl/guaiacyl ratio, molar mass, and thioacidolysis product yield than those in heterozygotes. Pretreatment with *Phanerochaete chrysosporium* achieved the highest delignification efficiency with a significant reduction in the cellulose content in both mulberry genotypes. In contrast, *Ceriporiopsis subvermispora* selectively removed lignin, with a weaker reduction in the cellulose content. The degree of delignification by *C. subvermispora* was significantly higher in homozygotes than in heterozygotes. *Trametes versicolor* tended to have a lower delignification capacity and smaller effect of subsequent enzymatic sugar release toward the wood from both genotypes than the other two fungi, making it less suitable for fungal pretreatment. Thioacidolysis assays indicated that cinnamaldehyde β-O-4, a typical subunit in the homozygote lignin, did not contribute to the high degradability of the lignin. The saccharification efficiency tended to be higher in homozygote wood than in heterozygote wood under all fungal pretreatment conditions.

Conclusions Although further optimization of various system conditions is required, our findings suggest that CAD deficiency promotes delignification and subsequent enzymatic saccharification and may improve the biorefining efficiency of wood when combined with fungal pretreatment.

Keywords Lignin mutant, Lignocellulose, Pretreatment, White rot fungus

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Background

Producing biofuels and biochemicals from renewable plant biomass may reduce society's dependence on fossil resources, particularly given the environmental and economic challenges of fossil resources. Lignocellulose, a major component of terrestrial plants, is widely available and chemically suitable as a feedstock in bio-based production. Efficient biorefinery processes are critical for optimal utilization of lignocellulose. The pretreatment step to partially separate lignocellulosic components, such as polysaccharides and lignin, is a major bottleneck in biorefining. Typical pretreatments involve mechanical, thermal, and chemical steps and/or combinations thereof. They often consume large amounts of energy and hazardous chemicals, require corrosion-resistant reactors, and produce large amounts of waste and compounds that limit subsequent processes.

From both economic and environmental perspectives, pretreatment with lignocellulolytic fungi may replace or, at least, supplement mechanical, thermal, or chemical pretreatment in lignocellulosic biorefineries [1]. White rot fungi (WRFs) are efficient degraders of polysaccharides and lignin in lignocellulosic biomass. They produce cellulases and hemicellulases as well as ligninolytic enzymes, such as laccase, manganese peroxidase, and lignin peroxidase. WRFs can be classified into two groups depending on the method by which they decompose materials [2]. Most WRFs simultaneously degrade polysaccharides and lignin [e.g., *Phanerochaete chrysosporium* (PC) and *Trametes versicolor* (TV)], resulting in homogenous cell wall decay. Among 26 basidiomycete strains tested in a previous study, PC showed the highest delignification rate of 51.4% in corn stover after pretreatment for 30 d, although it also showed the highest glucan loss [3]. Bamboo culms pretreated with TV strain G20 for 120 d showed a 12% decrease in the lignin content, leading to a 5.15-fold increase in sugar yield compared to that obtained using untreated material after saccharification [4]. In contrast, the WRF *Ceriporiopsis subvermispora* (CS) selectively degrades lignin without causing major damage to the cellulose in lignocellulose [5] and degrades lignin in cell walls and middle lamellae without eroding the wood cell walls [6]. Oxidation of small lipid molecules released extracellularly is thought to participate in the specific degradation mechanism [7]. Wan and Li [8, 9] reported that CS selectively degraded lignin in corn stover by up to 31.6% after 35 d, with a limited cellulose loss of < 6% during 18-d pretreatment. These differences in degradation are expected to have different effects on lignocellulosic materials in pretreatment and subsequent biorefining.

Lignin—the major chemical component responsible for the recalcitrance of lignocellulosic biomass [10]—hinders lignocellulose deconstruction and subsequent saccharification. It has three main aromatic substructures, namely *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), and has various different internal linkages, such as β-O-4, β-β, and β-5. Quantitative and/or qualitative modification of lignin can alter biomass recalcitrance. Many studies reported the successful modification of lignin via artificial regulation of biosynthetic genes, natural mutations, and genetic variations [11–16]. Some studies revealed that changes in the lignin content and structure contribute to biorefinery efficiency.

The combined effects of fungal pretreatment and lignin modification on reducing lignocellulosic recalcitrance has been evaluated in previous studies. Lignin in transgenic poplar overexpressing ferulate 5-hydroxylase, a key enzyme in biosynthesis of the S unit in lignin, comprised as much as 97.5% S units, in contrast to approximately 70% in wild-type poplar [17]. Despite the higher delignification efficiency of S-rich wood under alkaline pulping conditions [18], it was resistant to biodegradation when co-cultivated with both brown rot fungi and WRF for 4–16 weeks [19]. Giles et al. [20] also reported that lignin degradation in transgenic S-rich wood was inhibited compared with that in wild-type wood pretreated with CS, although the total weight loss was higher because of the accelerated degradation of polysaccharides. In contrast, in transgenic G-rich poplars with suppressed coniferaldehyde 5-hydroxylase activity, lignin was more easily degraded by the same fungal species than in wild-type poplars, leading to enhanced polysaccharide degradation [21]. The effect of fungal pretreatment on saccharification efficiency was evaluated in transgenic poplar wood under the individually suppressed expression of five lignin biosynthetic genes [21]. Pretreatment of transgenic poplars with CS downregulated the expression of cinnamate 3-hydroxylase, 4-coumarate:CoA ligase, caffeoyl-CoA O-methyltransferase, and coniferaldehyde 5-hydroxylase, yielding similar or decreased sugar release after enzymatic saccharification compared with that in non-fungal pretreatment controls.

Although various studies assessed the effects of fungal pretreatment on delignification and lignocellulose saccharification following lignin structure alteration, none have assessed the potential of fungal pretreatment combined with lignin-modified lignocellulose generated by transcriptional suppression or deletion of a gene encoding cinnamyl alcohol dehydrogenase (CAD). This enzyme catalyzes the conversion of hydroxycinnamaldehydes into their corresponding alcohols in the last step of monolignol biosynthesis. Suppression or loss-of-function of *CAD* promotes the incorporation of many of its substrates such as coniferaldehyde and sinapaldehyde into lignin as alternative lignin monomers [22, 23].

Cinnamaldehyde incorporation is occasionally accompanied by a decrease in the lignin content [24, 25] and partially alters three main lignin substructures bound through Cβ in the side chains of C6-C3 lignin skeletons, forming a β-O-4 linkage with an enol ether unit, a β-β linkage without a bicyclic furofuran ring, and a β-5 linkage with a benzofuran component [23, 26–29]. CAD deficiency improves the delignification and enzymatic saccharification efficiency of transgenic lignocelluloses and enhances the digestibility of forage crops [30–39]. Because of the excellent characteristics of CAD-deficient lignocellulose, we hypothesized that fungal pretreatment can accelerate the separation of its chemical components and increase the efficiency of subsequent biorefinery processes.

In this study, mulberry wood powder with an altered lignin structure was pretreated with three fungal species. Before evaluating the effects of lignin modification on delignification during fungal pretreatment and subsequent enzymatic saccharification, wood powders prepared from progeny plants with two different genotypes—homozygous (*cad*/*cad*) or heterozygous (*CAD*/*cad*) for the *cad*-null allele at the *CAD1* locus—were chemically characterized. This allele originated from the seed parent, a natural CAD mutant of mulberry (Sekizaisou), in which lignin is structurally altered [40]. The progenies were derived from a backcross between Sekizaisou and an F1 hybrid generated from Sekizaisou (seed parent) and Kokusou 21 (pollen parent). Our study suggests that fungal pretreatment of plants with genetically modified lignin structures greatly improves the efficiency of biomass biorefinery technology.

Results and discussion

Wood appearance of Sekizaisou progeny

Prior to fungal pretreatment and subsequent enzymatic saccharification, we characterized the wood of backcrossed progenies with different genotypes (*cad/cad* and *CAD/cad*). At harvest, the wood (xylem) under the bark had varying colors. After pulverization and sequential solvent extraction, the colors still differed, with *cad/cad* (homozygote) wood exhibiting a pale orange color, whereas *CAD/cad* (heterozygote) wood exhibited a light yellow (normal) color (Fig. 1). The colors of fresh and extractive-free homozygote wood and its extractive-free powder were similar to those of Sekizaisou, which also has a *cad/cad* genotype at the *CAD1* locus and an altered lignin structure [40]. The red wood phenotype is a typical characteristic and good indicator of CAD deficiency in mutant and transgenic plants [25, 41–43]. These findings confirm that the homozygous *cad*-null allele in the Sekizaisou progeny alters the wood color, a recessive trait, as reported in other plant species.

Chemical characterization of lignin in homozygote and heterozygote woods

The composition of the progeny wood was characterized using a combination of gravimetric and chemical analyses (Table 1). The Klason lignin (KL) content tended to be slightly lower in homozygotes than in heterozygotes ($P=0.11$). The acid-soluble lignin (ASL) content was 17% lower in the former than in the latter ($P<0.001$). These data are partially consistent with the low lignin content in Sekizaisou, as measured using the acetyl bromide method, compared with that in other mulberry varieties [40]. The KL content in 1-year-old stems of field-grown transgenic poplars with strong CAD suppression was also reported to be 10% lower than that in wild-type poplars [44].

The polysaccharide content of homozygotes and heterozygotes also differed. The cellulose content estimated from glucose released after acid hydrolysis of the wood powder was 12% lower than that of heterozygotes ($P<0.001$). In contrast, the hemicellulose content derived from the sum of four monosaccharides (mannose, galactose, xylose, and arabinose) tended to be higher in

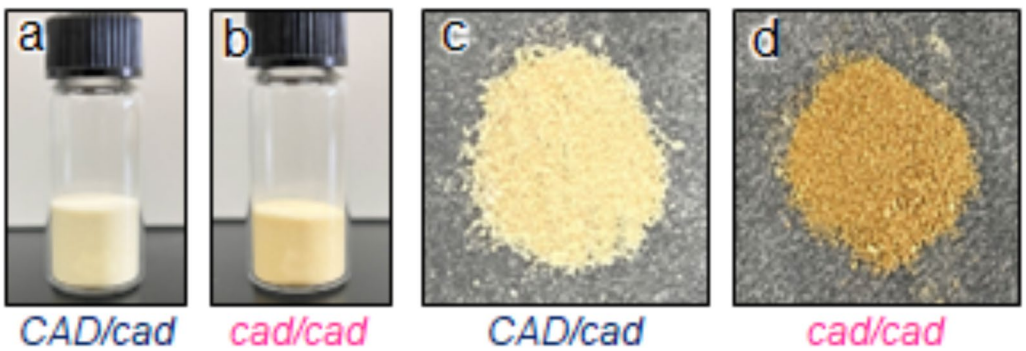

Fig. 1 Appearance of wood powders prepared from heterozygote (**a**, *CAD/cad*), homozygote (**b**, *cad/cad*), and cellulolytic enzyme lignins (**c**, **d**)

Table 1 Chemical composition of heterozygous (*CAD/cad*) and homozygous (*cad/cad*) samples

Progeny genotype	Chemical composition (dry weight %)			
	Klason lignin	Acid-soluble lignin	Cellulose	Hemicellulose
CAD/cad	24.9 ± 0.8	1.7 ± 0.0	51.7 ± 4.1	14.6 ± 1.0
cad/cad	22.2 ± 0.3**	1.4 ± 0.0**	47.4 ± 0.7	17.1 ± 0.7

** $P < 0.01$, compared with the heterozygote sample

Samples from three independent progenies of the same genotype were analyzed

homozygotes than in heterozygotes ($P = 0.054$). Sekizaisou, the seed parent of the progenies, also showed a lower cellulose (as glucose equivalent) content than did other mulberry varieties [38]. In a 1-year-old transgenic poplar with *CAD* suppression, the decrease in the lignin level was accompanied by a minor reduction in cellulose (average 3%) and small increase in hemicellulose levels (average 5%) compared with that in the wild-type plant [44]. Our results and those of previous studies indicate that a significant decrease in CAD activity leads to a slight decrease in the lignin content and change in the sugar composition in young woody plants.

Lignin monomeric composition

The monomeric composition of lignin was characterized using thioacidolysis (Table 2) [45, 46]. Calculations based on detection of conventional thioethylated C_6–C_3 monomers showed that the ratio of S to G units (S/G) in homozygous lignin (0.91) was significantly lower than that in heterozygous lignin (1.39). Similar trends were observed in other angiosperm species with CAD suppression [32, 47] as well as in a study comparing CAD-deficient Sekizaisou with other CAD-functional mulberry varieties [40]. Although the difference was not significant, the total amount of thioacidolysis monomers was 14% lower in the homozygous lignin than that in the heterozygous lignin.

CAD deficiency is commonly associated with the release of indene derivatives from the β-O-4 substructure coupled with cinnamaldehyde components in lignin under thioacidolysis [26, 29]. To confirm the changes in the lignin structure of the homozygotes, the thioacidolytic products were analyzed using gas chromatography/mass spectrometry (GC/MS). Four candidates were detected, three of which (1_{G1}, 1_{S1}, and 1_{S2}; Figure S1) were identified as indenes based on their mass spectra [40]. These compounds were also detected in thioacidolysis products prepared from Sekizaisou wood [40]. No indenes were detected in the heterozygote wood. The indenes could not be quantified in the present study because authentic compounds were not available; however, S-type indenes (1_{S1}, 1_{S2}, and with an asterisk in Figure S1) were clearly more abundant than was G-type indene (1_{G1}) (Figure S1). This result suggests that lignin in the homozygotes contains more β-O-4 substructure with sinapaldehyde unit than that with coniferaldehyde unit as reported previously in CAD-deficient Sekizaisou [40] and *CAD*-suppressed poplars [35]. If re-calculation is conducted to take this finding into consideration, the S/G ratio of homozygotes lignin is expected be even larger than the value (0.91) presented in Table 2. In addition to the colored wood phenotype, these findings confirm successful establishment of CAD-deficient progeny.

Total weight loss and sugar degradation under fungal pretreatment

For both genotypes of mulberry progeny, weight loss was greater in the order of pretreatment with PC, TV, and CS. Weight loss did not differ under PC, but homozygotes lost significantly more weight under TV and CS pretreatment than did heterozygotes (Table S1). These findings suggest that the change in lignin structure of

Table 2 Thioacidolysis monomer yields from heterozygotes (*CAD/cad*) and homozygotes (*cad/cad*)

Progeny genotype	Thioacidolytic monomer yields (μmol g^{-1} of lignin)			S/G ratio[a]
	G monomer	S monomer	Total	
CAD/cad	739.1 ± 59.4	1023.4 ± 70.9	1762.5 ± 129.8	1.39 ± 0.03
cad/cad	786.8 ± 11.1	718.4 ± 52.2*	1505.2 ± 56.0	0.91 ± 0.06**

[a] S:G monomer ratio

* $P < 0.05$, ** $P < 0.01$, compared to the heterozygote sample

Samples from three independent progenies of the same genotype were analyzed

homozygotes had a positive effect on degradation of the woods by these fungi under our experimental conditions.

In heterozygotes, cellulose degradation occurred in the order of PC > TV > CS, with significant differences between each sample. In homozygotes, the reduction in cellulose was also significant under PC, but its degradation was slower under TV and CS pretreatments, with no significant difference between them (Table S1). In both genotypes, cellulose degradation was the strongest contributor to weight loss under PC pretreatment (Tables 3 and S1). In contrast, weight loss in TV- and CS-pretreated wood was more affected by a reduction in the KL content than by polysaccharide degradation.

Delignification under fungal treatment

The KL levels in each wood sample after fungal pretreatment were more variable under PC and TV than under CS pretreatment, although cause of the variation was unclear. Regardless of the progeny genotype, the reduction in the wood fraction producing KL was likely larger under PC than that under TV and CS pretreatment (Tables 3 and S1), but the difference was not significant. Among the fungal pretreatments, only CS-treated homozygotes (27.6%) and heterozygotes (23.3%) differed in their KL degradation efficiencies ($P = 0.003$), suggesting that the altered lignin structure of the homozygote wood was more readily degraded by CS.

Although KL levels were generally lower in fungus-pretreated than in non-pretreated wood powders, the levels of most ASLs detected in both genotypes, except for the PC-treated homozygote wood, increased after fungal pretreatment. The increase in ASL levels was particularly pronounced in CS-treated homozygous wood ($P < 0.001$; Table 3). A substantial decrease in KL and increase in ASL levels were also observed in poplar wood disks [19] and aspen wood chips under CS degradation [48]. In addition, the increased rate of ASL was higher in homozygous wood after pretreatment with PC and TV than in heterozygous wood, although the difference was only significant following PC treatment ($P < 0.001$, Table S1). In non-pretreated wood, ASLs were partially composed of low-molecular weight lignin degradation products released by sulfuric acid hydrolysis [49]. The mechanism by which the acid solubility of lignin was increased under fungal degradation is unclear but was likely due to changes in the cleavage of lignin–polysaccharide and internal lignin linkages, a reduction in lignin molecular weight, and the introduction of more hydrophilic groups into lignin. Although ASL levels were significantly lower than those of KL, the remarkable increase in ASL released from CS- and PC-pretreated homozygous wood may reflect a partially unique manner of lignin decomposition.

Selective degradation of lignin

The selectivity index (SI, KL level reduction per unit of cellulose; Table S1) is an indicator of selective fungal degradation of lignin in lignocellulosic materials [50]. In heterozygous wood, the SI value was higher under CS (3.7) than under the other treatments ($P < 0.01$ for PC vs. CS and TV vs. CS), with no significant difference detected between PC (1.2) and TV (1.7). Similarly, the SI values for homozygous wood were in the decreasing order of PC (1.3), TV (1.2), and CS pretreatments (6.5), though no significant difference was detected among the genotypes because of the large variation in the SI value for CS pretreatment. These results suggest that, as previously reported [50], lignin-selective degradation by CS occurred in both heterozygous and homozygous wood.

Table 3 Chemical composition of wood powder from heterozygote (*CAD/cad*) and homozygote (*cad/cad*) before (WF) and after fungal pretreatment

Progeny genotype	Fungal strain	Chemical composition (wt.% of non-co-cultivated wood powder)[b]			
		Klason lignin	Acid-soluble lignin	Cellulose	Hemicellulose
CAD/cad	Without fungi[a]	24.9 ± 0.8	1.7 ± 0.0	51.7 ± 4.1	14.6 ± 1.0
	PC	17.8 ± 1.1^{x}	1.5 ± 0.0^{x}	40.7 ± 1.1^{x}	10.1 ± 1.2
	TV	19.9 ± 0.0^{y,z}	1.8 ± 0.0^{y}	47.8 ± 0.8^{y}	12.9 ± 0.2
	CS	19.1 ± 0.1^{x,z}	2.4 ± 0.1^{z}	50.8 ± 0.8^{z}	13.3 ± 1.0
cad/cad	Without fungi[a]	22.2 ± 0.3	1.4 ± 0.0	47.4 ± 0.7	17.1 ± 0.7
	PC	15.2 ± 1.4	1.4 ± 0.0^{x}	37.2 ± 2.0^{x}	12.6 ± 0.9^{x}
	TV	17.7 ± 1.3	1.6 ± 0.0^{y}	43.1 ± 0.5	14.4 ± 0.2^{y,z}
	CS	16.4 ± 0.5	2.4 ± 0.1^{z}	42.8 ± 2.8	13.9 ± 1.0^{x,z}

Samples from three or four independent progeny of the same genotype were analyzed

[a] Values are the same as those shown in Table 1

[b] Different letters indicate significant differences under pretreatment with different strains within the same progeny genotype ($P < 0.05$)

Characterization of lignin degradation by thioacidolysis

We further characterized lignin degradation under fungal pretreatment based on the release of monomeric products from fungus-degraded wood powders by thioacidolysis (Table 4). The levels of thioacidolytic monomers decreased in both wood genotypes after fungal pretreatment as the level of lignin decreased. Pretreatment of the heterozygote with PC and TV slightly reduced G and S monomer levels, but no significant differences were detected compared to those in non-pretreated samples. In contrast, the levels of each monomer and the total decreased significantly after CS pretreatment in the heterozygote. The pretreated homozygous wood showed a similar trend in monomer reduction as the heterozygous wood; however, there was no significant difference between the different fungal strains in terms of S monomer reduction. The lowest monomer level was observed in CS-pretreated homozygous wood, suggesting a more condensed structure with few β-O-4 linkages.

A slight difference in the S/G ratio was observed among the genotypes under different fungal pretreatments, and most values were comparable before and after fungal degradation. Although preferential degradation of S units was reported in the lignin of wheat straw and oak wood under CS decomposition [16, 51] it was not observed in the present study.

The thioacidolytic dimer products after desulfurization were analyzed using GC/MS [52, 53]. We identified 18 different dimers based on their mass spectra, of which 5, 4, 3, 3, and 3 compounds had β-5, β-1, β-β, 4-O-5, and 5–5 substructures, respectively. Seven compounds consisted of two G units (GG), four had two S units (SS), and seven had both S and G units (SG). The total level of dimers detected in non-pretreated homozygous wood was 11% lower than that in heterozygous wood (Fig. 2).

Table 4 Thioacidolysis monomer yields from fungus-pretreated heterozygote (*CAD/cad*) and homozygote (*cad/cad*) wood

Progeny genotype	Fungal strain	Thioacidolytic monomer yields (μmol g^{-1} of lignin)			S/G ratio
		G monomer	S monomer	Total	
CAD/cad	Without fungi[a]	739 ± 59^{x}	1023 ± 71^{x}	1762 ± 130^{x}	1.39 ± 0.03^{x}
	PC	666 ± 40^{x}	842 ± 41^{x}	1508 ± 81^{x}	1.26 ± 0.01^{y}
	TV	657 ± 24^{x}	896 ± 44^{x}	1552 ± 68^{x}	1.36 ± 0.02^{x}
	CS	440 ± 6^{y}	612 ± 8^{y}	1052 ± 14^{y}	1.39 ± 0.00^{x}
cad/cad	Without fungi[a]	787 ± 11^{x}	718 ± 52^{x}	1505 ± 56^{x}	0.91 ± 0.06^{x}
	PC	629 ± 53^{x}	$556 \pm 76^{x,y}$	$1186 \pm 129^{x,y}$	0.88 ± 0.05^{x}
	TV	658 ± 40^{x}	$606 \pm 65^{x,y}$	1264 ± 105^{x}	0.92 ± 0.05^{x}
	CS	400 ± 39^{y}	$387 \pm 44^{y,z}$	787 ± 82^{y}	0.97 ± 0.04^{x}

[a] Values are the same as those shown in Table 1

[b] Different letters indicate significant differences under pretreatment with different strains within the same progeny genotype ($P < 0.05$)

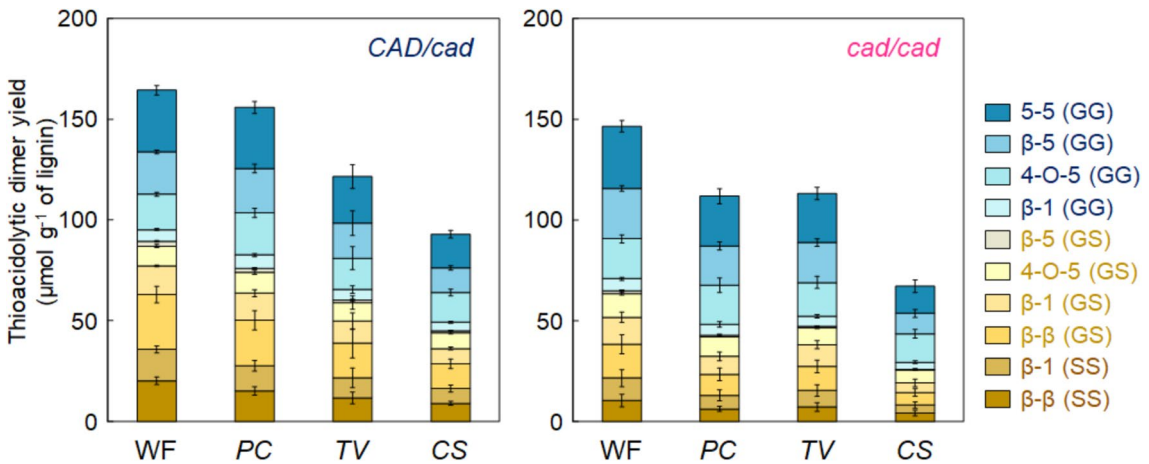

Fig. 2 Thioacidolysis dimer yields of heterozygote (left) and homozygote (right) woods before (WF, without fungi) and after fungal pretreatment. PC, *P. chrysosporium*. TV, *T. versicolor*. CS, *C. subvermispora*

The low yield of total thioacidolytic monomers and dimers in the homozygote suggest that the homozygote lignin has a more "condensed (rich in carbon–carbon bond)" structure than the heterozygote lignin, as was also reported in CAD-deficient transgenic poplar [54]. The relative proportion of β-β-type dimers, in which the structure with two S units predominates, was markedly lower in the non-pretreated homozygote than in the heterozygote. The relative compositions of the GG, SS, and SG dimers were 55.8%, 14.6%, and 29.6% in homozygotes, whereas they were 45.6%, 21.8%, and 32.6% in heterozygotes, respectively. The lower proportion of dimers containing S units in the homozygote was consistent with the lower S/G ratio calculated from the lignin monomers.

After fungal treatment, the total amount of dimers was reduced to 5–43% in heterozygotes and 23–54% in homozygotes, suggesting that homozygote lignin is more easily degraded than is heterozygote lignin. The most abundant dimers detected after fungal treatment had the G(5–5)G substructure in both genotypes, regardless of the fungal species, indicating that the lignin substructure with the 5–5 linkage is difficult for fungi to degrade. The largest decrease in dimer levels after fungal degradation was observed in homozygous wood under CS pretreatment (28% decrease compared with that in heterozygous wood).

Effect of β-O-4 interunit coupling with cinnamaldehyde units on fungal degradation of lignin

As previously reported [40], CAD deficiency in Sekizaisou generated abnormal β-O-4 subunits with cinnamaldehyde residue in its lignin. These structures may contribute to delignification in CAD-deficient woods under alkaline conditions [31, 38, 44]. To investigate their contribution to the fungal degradation of homozygous wood, the amounts of indene derivatives released by thioacidolysis of the degraded wood were compared based on their peak area of the mass chromatograms (Figure S1) [26, 29]. The levels of the three indenes increased slightly after fungal degradation but were not correlated with the reduction in total weight or lignin content. These results indicate that the abnormal β-O-4 substructure does not affect the degradability of homozygous wood and its lignin.

Lignin molecular distribution before and after fungal pretreatment

Size exclusion chromatography was used to compare the molar mass distribution of cellulolytic enzyme lignins (CELs) prepared from heterozygous and homozygous wood before and after fungal pretreatment. CEL prepared from non-pretreated homozygous wood showed smaller weight-average molar mass (Mw) and number-average molar mass (Mn) values than that from non-pretreated heterozygous wood (Fig. 3 and Table S2). These results suggest that CAD deficiency, the associated changes in the composition of lignin monomers, and resultant substructures inhibit elongation of the lignin chain in the cell wall, thereby at least partially contributing to the accelerated delignification of homozygous wood during fungal pretreatment. A decrease in the Mw of lignin was also reported for CAD-deficient transgenic poplar [54], *Arabidopsis*, and loblolly pine mutants [55, 56], in which delignification of lignocellulosic materials was improved under deep eutectic solvent extraction or alkaline pulping [31, 36, 55].

The Mw was further decreased after fungal pretreatment, with the reduction rate roughly correlated with the delignification efficiency of each fungus, regardless of the genotype (Figure S2 and Table S2). The largest decrease in the CEL Mw was observed in both genotypes under PC, which is consistent with the largest reduction in KL levels under the same pretreatment. The reduction in CEL Mw after PC pretreatment was greater in homozygotes (35%) than in heterozygotes (27%) (Table S2). This may be partially due to the slightly higher delignification efficiency of the former (Table S2). Mw reduction was also detected in CS-degraded homozygous and heterozygous CELs; however, unlike under PC, the reduction rate was higher in heterozygotes (37%) than in homozygotes (24%). In contrast to PC and CS, there was no apparent difference in the Mw reduction rate between the two genotypes after TV pretreatment. The smaller CEL Mw in

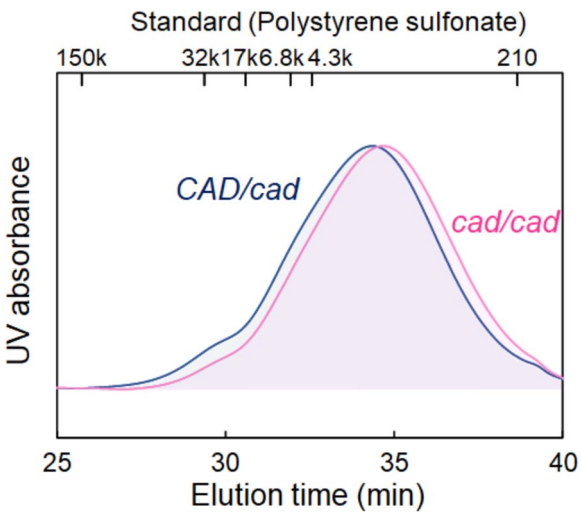

Fig. 3 Molar mass distribution of cellulolytic enzyme lignins (CELs) from non-pretreated homozygotes (*cad/cad*) and heterozygotes (*CAD/cad*)

the non-pretreated homozygous wood should benefit the fungal degradation of lignin compared to that in the heterozygous wood.

Combined effect of lignin alteration and fungal pretreatment on saccharification efficiency

Saccharification efficiency was calculated as the amount of glucose released by enzymatic hydrolysis of wood powder with and without fungal pretreatment. The saccharification efficiency of the CS-treated heterozygous wood, based on the glucose units in the pretreated wood estimated as cellulose (78%), was higher than that under PC (60%, $P=0.07$) and TV pretreatment (53%, $P=0.017$) and without fungal pretreatment (62%, $P=0.11$). In homozygous wood, the saccharification efficiency was greatest after CS pretreatment ($P<0.001$, compared to TV and non-fungal pretreatment), whereas the difference under PC pretreatment was less pronounced ($P=0.055$). Collectively, CS pretreatment contributed to improved glucose release from both mulberry genotypes, despite the reduction in cellulose during fungal pretreatment.

In contrast to the differences in fungal pretreatments, glucose yield did not significantly differ between the different genotypes, except for in PC-pretreated woods (Fig. 4). However, after pretreatment with TV and PC, the yield tended to be higher in homozygotes than in heterozygotes. As backcross progeny plants (*CAD/cad* and *cad/cad*) were generated by artificial crossing of Sekizaisou (*cad/cad*) and its F1 progeny (*CAD/cad*), their genotypes may vary greatly at some gene loci, even if individual plants had the same genotype at the *CAD* locus. Furthermore, as pretreatment was performed under semi-solid culture with wood powder, the fungal mycelia likely grew nonuniformly. Therefore, the genetic diversity of the wood samples and non-uniform fungal culture conditions in the present study may have limited the detection of differences between the mulberry genotypes under TV and CS pretreatments.

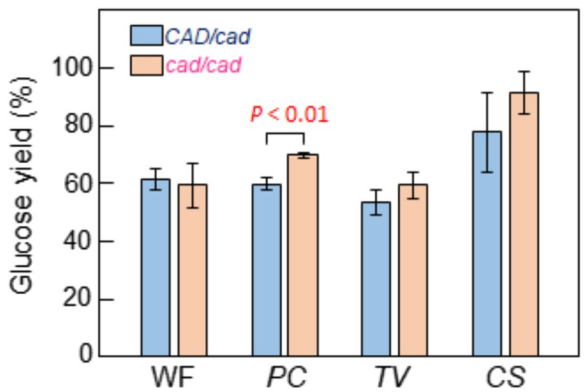

Fig. 4 Glucose yields from heterozygotes (*CAD/cad*) and homozygotes (*cad/cad*) with and without (WF) fungal pretreatment, PC, *P. chrysosporium*. TV, *T. versicolor*. CS, *C. subvermispora*. Data were analyzed using Student's *t*-test ($n=3$)

Conclusions

Our in-depth analysis of lignin modification and fungal degradation in two genotypically different mulberries showed that genetic modifications at the *CAD1* locus had a significant effect on the lignin content, chemical composition, CEL molar mass, and fungal degradation of the wood. We found that wood deficient in CAD showed improved enzymatic saccharification when combined with fungal pretreatment. Saccharification efficiency is influenced by a variety of factors, including the species of fungi and pretreatment conditions including temperature, moisture, nutrients, and lignocellulose size. Thus, optimization of these factors may further improve the biorefining efficiency of lignin-modified wood.

Materials and methods

Plant material

Wood samples were prepared from the branching stems of backcross progenies between a *cad*-null mutant Sekizaisou (*cad*/*cad* at *CAD1* locus, seed parent) and F1 hybrid (line #8, *CAD*/*cad*, pollen parent). The latter was derived from a cross between Sekizaisou and another mulberry variety, Kokusou 21 (*CAD*/*CAD*), as seed and pollen parents, respectively. The backcross progenies were categorized as heterozygotes (*CAD*/*cad*) or homozygotes (*cad*/*cad*) based on their genotypes for the *cad*-null allele at the *CAD1* locus. On March 27, 2021, 45 heterozygote and 49 homozygote seedlings were planted in an experimental field (35°58′31.8″ N, 140°04′57.2″ E). Before planting, the genotype of each individual was confirmed by PCR using total DNA and Sanger sequencing of PCR-amplified DNA, as previously reported [57]. The branching stems of 10 well-grown individual plants of each genotype were harvested separately on February 25, 2022, of which three were used in this study. As in Sekizaisou, the xylem in all homozygotes exhibited a bright red color in summer and pale pink color at harvest. In contrast, the xylem of the heterozygote showed a normal pale yellowish-white color.

Preparation of wood powder

The branched stems were debarked using a hand knife and air-dried at room temperature for several days. The air-dried debarked stems were cut into small pieces (approximately 1 cm in length) using a band saw (U-32, LUXO, Nagoya, Japan). The wood chips were subjected to ground and pulverized using a Wiley and Rotor Speed mill (PULVERISETTE 14, FRITSCH, Yokohama, Japan), and powders were obtained after filtration through a

150-μm mesh. The pulverized wood samples were subjected to solvent extraction to obtain an extractive-free wood powder as previously described [58].

Pre-cultivation of white rot fungi

Three strains of white rot fungus (Figure S2), *C. subvermispora*, *P. chrysosporium*, and *T. versicolor*, were cultivated on potato dextrose agar medium for 7 d at 28 °C prior to the biodegradation experiment. The surface of the mycelium-containing potato dextrose agar was punched using a flame-sterilized stainless steel cork borer (diameter of approximately 6.0 mm) to prepare fungal discs for use in the degradation experiment.

Fungal degradation experiment

Distilled water was added to 300 mg wood powder from homozygotes or heterozygotes in a 50-mL volumetric flask to a final water content of 62.5%. All flasks were covered with a silicon cap and foil to prevent water evaporation and autoclaved at 121 °C for 20 min. Each flask was inoculated with one fungal disc from a 7-d-old culture and incubated for 14 d at 28 °C. An open container with water was placed in the incubator to maintain a high humidity level. A flask containing sterile un-inoculated wood powder (without fungi) was used as a control. The experiment was performed using three or four biological samples from each genotype. Fungus-degraded wood powders were collected after 14 d of pretreatment, vacuum-filtrated using a pre-weighed PTFE membrane filter (pore size 0.45 μm), and oven-dried at 60 °C after being thoroughly cleaned with distilled water to remove fungal mycelia.

Klason and acid-soluble lignins

Wood powder (approximately 20 mg) with and without fungal pretreatment was hydrolyzed in 72% sulfuric acid for 1 h at room temperature and autoclaved in 3% sulfuric acid at 121 °C for 1 h. After cooling, the solution was filtered through a pre-weighed PTFE membrane filter. Solids on the filter were dehydrated at 60 °C overnight and weighed as KLs. For ASL quantification, the absorbance of the filtered solution was measured at 205 nm and estimated using the absorption coefficient (110 g^{-1} L cm^{-1}).

Thioacidolysis

To assess the monomeric composition of lignin in wood powders with and without fungal pretreatment, thioacidolysis was performed as previously described [53]. After trimethylsilylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide in pyridine, the thioacidolytic products were separated using a gas chromatograph (GC-2010 Plus, Shimadzu Corporation, Kyoto, Japan) equipped with a flame-ionization detector. Conventional trithioethylated C6-C3 monomers were quantified using the response factors reported by Yue et al. [46]. Indenes, indicators of CAD-deficient lignin released by thioacidolysis [26, 29], were identified using a GC/MS.

Thioacidolysis and subsequent Raney nickel desulfurization of the products were performed to evaluate the interunit linkages estimated from the thioacidolytic dimer composition [59, 60]. GC/MS analysis of the dimeric compounds was performed using a GC-2010 system (Shimadzu). Helium was used as the carrier gas, and the trimethylsilylated sample was separated over an RTx-5MS column (GL Science, Tokyo, Japan; length: 30 m × 0.25 mm i.d., linear velocity: 47 cm/s in spitless mode). The column temperature was maintained at 180 °C for 1 min, increased to 280 °C at 2 °C/min, and maintained for 9 min. The temperatures of the inlet and ion sources were set to 250 °C and 260 °C, respectively. The yield of each eluted dimer was calculated using an assumed response factor of 1.5 to tetracosane [53].

Monosaccharide composition

The monosaccharide composition of wood powder with and without fungal pretreatment was determined as previously described [61].

Size exclusion chromatography of CEL

The molar mass distribution of CEL prepared from extractive-free wood powder with and without fungal pretreatment was performed by size exclusion chromatography according to the procedures used in previous studies [62, 63]. To prepare the CELs, the wood powders were ball-milled at 600 rpm for 3 cycles of 10 min each and 15-min intervals to avoid overheating. The ball-milled samples (150–200 mg) were suspended in citric acid buffer (38 mL, pH 4.8) in 50-mL tubes and incubated for 24 h at room temperature. After 24 h, the tubes were treated with three enzymes (per 1 g of wood powder): 4.32 mL Cellic CTec2 (11.6 mg/mL, SAE0020, Sigma-Aldrich, St. Louis, MO, USA), 3 mL β-glucosidase (5 mg/mL, X2753, Sigma-Aldrich), 3 mL D-glucosidase (5 mg/mL, 49,290, Sigma-Aldrich), and 1 mL Tween-20. The mixture-containing tubes were subjected to enzymatic saccharification at 50 °C for 72 h with inverted mixing. After saccharification, the residues were collected by washing with distilled water three times and vacuum-filtered using 47-mm filter paper (0.45 μm PTFE membrane, Omnipore, Merck, Kenilworth, NJ, USA). The collected residues were freeze-dried overnight and extracted with 96% dioxane (v/v) for 24 h with continuous stirring. The extraction procedure was performed twice. Lignin in the 96% dioxane extract was purified by rotary evaporation and precipitated in acidic water (pH 2.0) overnight. The precipitate was washed with distilled

water, collected by vacuum filtration, and freeze-dried. The prepared CEL was dissolved in 10 mM lithium bromide-dimethyl sulfoxide solution at a concentration of 0.24 mg/mL and filtered through a membrane filter (DISMIC-13 HP PTFE, Advantec, Dublin, CA, USA). The filtrate was analyzed using high-performance liquid chromatography (Chromaster, HITACHI, Tokyo, Japan) equipped with two PolarGel M columns (7.5×300 mm, Agilent Technologies, Santa Clara, CA, USA) and a guard column (7.5×50 mm) under the following conditions: flow rate of 0.5 mg/mL, column temperature of 50 °C, and UV detector at 280 nm.

Saccharification efficiency

The wood powders with and without fungal pretreatment (5 mg) were subjected to enzymatic saccharification in 5 mM citrate buffer (pH 4.8) with celluclast (C2730, Sigma-Aldrich), cellobiase (C6105, Sigma-Aldrich), and 0.02% sodium azide at 50 °C for 24 h with inverted mixing [58]. Released glucose was detected as its alditol acetate derivative and quantified using a GC-flame-ionization detector. Saccharification efficiencies were calculated based on total cellulose in the non-pretreated or fungal-pretreated wood and released glucose (cellulose equivalent) after saccharification. All analyses were performed with three biological replicates for each genotype.

Statistical analysis

All data were analyzed using Student's *t*-test or Tukey's multiple comparison test.

Abbreviations

ASL	Acid-soluble lignin
CEL	Cellulolytic enzyme lignin
CS	*Ceriporiopsis subvermispora*
CAD	Cinnamyl alcohol dehydrogenase gene
GC/MS	Gas chromatography/mass spectrometry
G	Guaiacyl
KL	Klason lignin
Mw	Weight-average molar mass
Mn	Number-average molar mass
PC	*Phanerochaete chrysosporium*
H	*p*-Hydroxyphenyl
S	Syringyl
TV	*Trametes versicolor*
WRFs	White rot fungi

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-025-02611-y.

Additional file 1.

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Authors contribution

MTakada and SK conceptualized and designed the experiments; JPTM and MTerasaki conducted the experiments; JPTM, MTakada, and SK interpreted the data; JPTM wrote the original draft; JPTM, MTakada, and SK wrote, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

1. Gupta R, Mehta G, Khasa YP, Kuhad RC. Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics. Biodegradation. 2011;22(4):797–804.
2. Fernandez-Fueyo E, Ruiz-Duenas FJ, Ferreira P, Floudas D, Hibbett DS, Canessa P, et al. Comparative genomics of *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* provide insight into selective ligninolysis. Proc Natl Acad Sci USA. 2012;109(14):5458–63.
3. Saha BC, Qureshi N, Kennedy GJ, Cotta MA. Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis. Int Biodeter Biodegr. 2016;109:29–35.
4. Zhang XY, Yu HB, Huang HY, Liu YX. Evaluation of biological pretreatment with white rot fungi for the, enzymatic hydrolysis of bamboo culms. Int Biodeter Biodegr. 2007;60(3):159–64.
5. Tanaka H, Koike K, Itakura S, Enoki A. Degradation of wood and enzyme production by *Ceriporiopsis subvermispora*. Enzyme Microb Tech. 2009;45(5):384–90.
6. Messner K, Srebotnik E. Biopulping—an overview of developments in an environmentally safe paper-making technology. Fems Microbiol Rev. 1994;13(2–3):351–64.
7. Enoki M, Watanabe T, Nakagame S, Koller K, Messner K, Honda Y, et al. Extracellular lipid peroxidation of selective white-rot fungus. Fems Microbiol Lett. 1999;180(2):205–11.
8. Wan CX, Li YB. Microbial delignification of corn stover by *Ceriporiopsis subvermispora* for improving cellulose digestibility. Enzyme Microb Tech. 2010;47(1–2):31–6.

9. Wan CX, Li YB. Microbial pretreatment of corn stover with *Ceriporiopsis subvermispora* for enzymatic hydrolysis and ethanol production. Bioresource Technol. 2010;101(16):6398–403.
10. Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, et al. Lignin content in natural *Populus* variants affects sugar release. Proc Natl Acad Sci USA. 2011;108(15):6300–5.
11. Sattler SE, Funnell-Harris DL, Pedersen JF. Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. Plant Sci. 2010;178(3):229–38.
12. Van Acker R, Vanholme R, Storme V, Mortimer JC, Dupree P, Boerjan W. Lignin biosynthesis perturbations affect secondary cell wall composition and saccharification yield in *Arabidopsis thaliana*. Biotechnol Biofuels. 2013;6(1):46.
13. Vermerris W, Abril A. Enhancing cellulose utilization for fuels and chemicals by genetic modification of plant cell wall architecture. Curr Opin Biotech. 2015;32:104–12.
14. Mottiar Y, Vanholme R, Boerjan W, Ralph J, Mansfield SD. Designer lignins: harnessing the plasticity of lignification. Curr Opin Biotechnol. 2016;37:190–200.
15. Bryant ND, Pu Y, Tschaplinski TJ, Tuskan GA, Muchero W, Kalluri UC, et al. Transgenic poplar designed for biofuels. Trends Plant Sci. 2020;25(9):881–96.
16. van Erven G, Hendrickx P, Al Hassan M, Beelen B, op den Kamp R, Keijsers E, et al. Plant genotype and fungal strain harmonization improves *Miscanthus sinensis* conversion by the white-rot fungus *Ceriporiopsis subvermispora*. Acs Sustain Chem Eng. 2023;11(17):6752–64.
17. Stewart JJ, Akiyama T, Chapple C, Ralph J, Mansfield SD. The effects on lignin structure of overexpression of ferulate 5-hydroxylase in hybrid poplar. Plant Physiol. 2009;150(2):621–35.
18. Huntley SK, Ellis D, Gilbert M, Chapple C, Mansfield SD. Significant increases in pulping efficiency in C4H–F5H-transformed poplars: improved chemical savings and reduced environmental toxins. J Agr Food Chem. 2003;51(21):6178–83.
19. Skyba O, Douglas CJ, Mansfield SD. Syringyl-rich lignin renders poplars more resistant to degradation by wood decay fungi. Appl Environ Microbiol. 2013;79(8):2560–71.
20. Giles R, Peszlen I, Peralta P, Chang HM, Farrell R, Grand L, et al. Fungal biodegradation of genetically modified and lignin-altered quaking aspen (*Populus tolemuroides* Michx.). Holzforschung. 2012;66(1):105–10.
21. Edmunds CW, Peralta P, Sharma-Shivappa RR, Kelley SS, Chiang VL, Miller ZD, et al. Fungal pretreatment and enzymatic hydrolysis of genetically-modified *Populus trichocarpa*. BioResources. 2020;15(3):6488–505.
22. Stewart D, Yahiaoui N, McDougall GJ, Myton K, Marque C, Boudet AM, et al. Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamaldehydes into the lignin of transgenic tobacco (*Nicotiana tabacum* L.) plants with reduced expression of cinnamyl alcohol dehydrogenase. Planta. 1997;201(3):311–8.
23. Ralph J, Hatfield RD, Piquemal J, Yahiaoui N, Pean M, Lapierre C, et al. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamylalcohol dehydrogenase and cinnamoyl-CoA reductase. Proc Natl Acad Sci USA. 1998;95(22):12803–8.
24. Sibout R, Eudes A, Mouille G, Pollet B, Lapierre C, Jouanin L, et al. *CINNAMYL ALCOHOL DEHYDROGENASE-C* and *-D* are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis*. Plant Cell. 2005;17(7):2059–76.
25. Zhao Q, Tobimatsu Y, Zhou R, Pattathil S, Gallego-Giraldo L, Fu C, et al. Loss of function of cinnamyl alcohol dehydrogenase 1 leads to unconventional lignin and a temperature-sensitive growth defect in *Medicago truncatula*. Proc Natl Acad Sci USA. 2013;110(33):13660–5.
26. Kim H, Ralph J, Lu F, Pilate G, Leple JC, Pollet B, et al. Identification of the structure and origin of thioacidolysis marker compounds for cinnamyl alcohol dehydrogenase deficiency in angiosperms. J Biol Chem. 2002;277(49):47412–9.
27. Kim H, Ralph J, Lu F, Ralph SA, Boudet AM, MacKay JJ, et al. NMR analysis of lignins in CAD-deficient plants. Part 1. Incorporation of hydroxycinnamaldehydes and hydroxybenzaldehydes into lignins. Org Biomol Chem. 2003;1(2):268–81.
28. Yoshioka K, Kim H, Lu F, De Ridder N, Vanholme R, Kajita S, et al. Hydroxycinnamaldehyde-derived benzofuran components in lignins. Plant Physiol. 2024;194(3):1370–82.
29. Lapierre C, Pilate G, Pollet B, Mila I, Leple JC, Jouanin L, et al. Signatures of cinnamyl alcohol dehydrogenase deficiency in poplar lignins. Phytochem. 2004;65(3):313–21.
30. Barriere Y, Argillier O, Chabbert B, Tollier MT, Monties B. Breeding silage maize with brown-midrib genes—feeding value and biochemical characteristics. Agronomie. 1994;14(1):15–25.
31. Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, et al. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant Physiol. 1999;119(1):153–64.
32. Baucher M, Bernard-Vailhe MA, Chabbert B, Besle JM, Opsomer C, Van Montagu M, et al. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the effect on lignin composition and digestibility. Plant Mol Biol. 1999;39(3):437–47.
33. Chen L, Auh CK, Dowling P, Bell J, Chen F, Hopkins A, et al. Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. Plant Biotechnol J. 2003;1(6):437–49.
34. Anderson NA, Tobimatsu Y, Ciesielski PN, Ximenes E, Ralph J, Donohoe BS, et al. Manipulation of guaiacyl and syringyl monomer biosynthesis in an Arabidopsis cinnamyl alcohol dehydrogenase mutant results in atypical lignin biosynthesis and modified cell wall structure. Plant Cell. 2015;27(8):2195–209.
35. Van Acker R, Dejardin A, Desmet S, Hoengenaert L, Vanholme R, Morreel K, et al. Different routes for conifer- and sinapaldehyde and higher saccharification upon deficiency in the dehydrogenase CAD1. Plant Physiol. 2017;175(3):1018–39.
36. Kim KH, Eudes A, Jeong K, Yoo CG, Kim CS, Ragauskas A. Integration of renewable deep eutectic solvents with engineered biomass to achieve a closed-loop biorefinery. Proc Natl Acad Sci USA. 2019;116(28):13816–24.
37. Liu X, Van Acker R, Voorend W, Pallidis A, Goeminne G, Pollier J, et al. Rewired phenolic metabolism and improved saccharification efficiency of a *Zea mays cinnamyl alcohol dehydrogenase 2* (*zmcad2*) mutant. Plant J. 2021;105(5):1240–57.
38. Ikeda T, Takata N, Sakamoto S, Hu S, Nuoendagula N, Hishiyama S, et al. Improved chemical pulping and saccharification of a natural mulberry mutant deficient in cinnamyl alcohol dehydrogenase. Holzforschung. 2021;75(10):968–77.
39. Martin AF, Tobimatsu Y, Lam PY, Matsumoto N, Tanaka T, Suzuki S, et al. Lignocellulose molecular assembly and deconstruction properties of lignin-altered rice mutants. Plant Physiol. 2023;191(1):70–86.
40. Yamamoto M, Tomiyama T, Koyama A, Okuizumi H, Liu S, Vanholme R, et al. A century-old mystery unveiled: Sekizaisou is a natural lignin mutant. Plant Physiol. 2020;182(4):1821–8.
41. Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, et al. Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. Plant J. 1994;6(3):339–50.
42. Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier MT, Petit-Conil M, et al. Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiol. 1996;112(4):1479–90.
43. Barrière Y, Chavigneau H, Delaunay S, Courtial A, Bosio M, Lassagne H, et al. Different mutations in the *ZmCAD2* gene underlie the maize brown-midrib1 (*bm1*) phenotype with similar effects on lignin characteristics and have potential interest for bioenergy production. Maydica. 2013;58(1):6–20.
44. De Meester B, Van Acker R, Wouters M, Traversari S, Steenackers M, Neukermans J, et al. Field and saccharification performances of poplars severely downregulated in *CAD1*. New Phytol. 2022;236(6):2075–90.
45. Lapierre C. Application of new methods for the investigation of lignin structure. In: Jung HG, Buxton DR, Hatfield RD, Ralph J, editors. Forage cell wall structure and digestibility. Madison: American Society of Agronomy; 1993. p. 133–66.
46. Yue F, Lu F, Sun RC, Ralph J. Syntheses of lignin-derived thioacidolysis monomers and their uses as quantitation standards. J Agric Food Chem. 2012;60(4):922–8.
47. Vailhé MAB, Besle JM, Maillot MP, Cornu A, Halpin C, Knight M. Effect of down-regulation of cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. J Sci Food Agr. 1998;76(4):505–14.

48. Choi JW, Choi DH, Ahn SH, Lee SS, Kim MK, Meier D, et al. Characterization of trembling aspen wood (*Populus tremuloides* L.) degraded with the white rot fungus and MWLs isolated thereof. Holz Roh Werkst. 2006;64(5):415–22.
49. Swan B. Isolation of acid-soluble lignin from the Klason lignin determination. Svensk Papperstidn. 1965;68(22):791–5.
50. Chi Y, Hatakka A, Maijala P. Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? Int Biodeterior Biodegrad. 2007;59(1):32–9.
51. van Kuijk SJA, Sonnenberg ASM, Baars JJP, Hendriks WH, del Rio JC, Rencoret J, et al. Chemical changes and increased degradability of wheat straw and oak wood chips treated with the white rot fungi *Ceriporiopsis subvermispora* and *Lentinula edodes*. Biomass Bioenerg. 2017;105:381–91.
52. Lapierre C, Pollet B, Monties B, Rolando C. Thioacidolysis of spruce lignin—GC–Ms analysis of the main dimers recovered after Raney-nickel desulfuration. Holzforschung. 1991;45(1):61–8.
53. Kishimoto T, Chiba W, Saito K, Fukushima K, Uraki Y, Ubukata M. Influence of syringyl to guaiacyl ratio on the structure of natural and synthetic lignins. J Agric Food Chem. 2010;58(2):895–901.
54. Baumberger S, Dole P, Lapierre C. Using transgenic poplars to elucidate the relationship between the structure and the thermal properties of lignins. J Agric Food Chem. 2002;50(8):2450–3.
55. Dimmel DR, MacKay JJ, Althen EM, Parks C, Sederoff RR. Pulping and bleaching of CAD deficient wood. J Wood Chem Technol. 2001;21(1):1–17.
56. Jacquet N, Eudes A, Dutta T, Kim KH, Bouxin F, Benites V, et al. Influence of hydrocracking and ionic liquid pretreatments on composition and properties of *Arabidopsis thaliana* wild type and CAD mutant lignins. Renew Energy. 2020;152:1241–9.
57. Hachisuka H, Fukuda S, Iwase M, Inagaki S, Tomiyama H, Okuizumi H, et al. Detection protocol for a mutant allele on the *CINNAMYL ALCOHOL DEHYDROGENASE 1* locus of the *Morus* species and search trial for the allele in the natural mulberry population of Okushiri island. Lignin. 2020;1:42–50.
58. Hu S, Kamimura N, Sakamoto S, Nagano S, Takata N, Liu S, et al. Rerouting of the lignin biosynthetic pathway by inhibition of cytosolic shikimate recycling in transgenic hybrid aspen. Plant J. 2022;110(2):358–76.
59. Lapierre C, Pollet B, Rolando C. New insights into the molecular architecture of hardwood lignins by chemical degradative methods. Res Chem Intermediat. 1995;21(3–5):397–412.
60. Yue F, Lu F, Regner M, Sun R, Ralph J. Lignin-derived thioacidolysis dimers: reevaluation, new products, authentication, and quantification. Chemsuschem. 2017;10(5):830–5.
61. Blakeney AB, Harris PJ, Henry RJ, Stone BA. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohyd Res. 1983;113(2):291–9.
62. Chang HM, Cowling EB, Brown W, Adler E, Miksche G. Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. Holzforschung. 1975;29(5):153–9.
63. Hu ZJ, Yeh TF, Chang HM, Matsumoto Y, Kadla JF. Elucidation of the structure of cellulolytic enzyme lignin. Holzforschung. 2006;60(4):389–97.

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