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A functional cascading of lignin modification via repression of caffeic acid *O*-methyltransferase for bioproduction and anti-oxidation in rice

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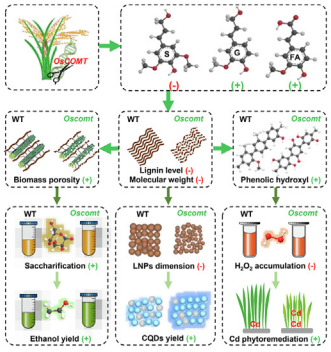
HIGHLIGHTS

- *OsCOMT* knock-out mutants show slightly altered plant growth and biomass yield.
- Improved anti-oxidation capacity for heavy-metal phytoremediation in the mutants.
- Remarkably enhanced biomass saccharification for high-yield bioethanol production.
- Modified lignin favors for smaller nanoparticles and high-yield carbon quantum dots.
- A novel mechanism about one-gene mutation for improving biofuels, bioproducts and antioxidation.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Crop straws provide substantial biomass resources that are transformable for sustainable biofuels and valuable bioproducts. However, the natural lignocellulose recalcitrance results in an expensive biomass process and secondary waste liberation. As lignin is a major recalcitrant factor, genetic engineering of lignin biosynthesis is increasingly being implemented in bioenergy crops, but much remains unclear about the desired lignocellulose alteration and resulting function.
Objectives: This study attempted to explore the mechanisms of lignin modification responsible for efficient lignocellulose conversion *in vitro* and an effective plant anti-oxidation response *in vivo*.
Methods: We initially selected specific rice mutants by performing modern CRISPR/cas9 editing with caffeic acid *O*-methyltransferase involved in the synthetic pathways of monolignols (G, S) and ferulic acid (FA), and then explored lignocellulose conversion and plant cadmium (Cd) accumulation using advanced chemical, biochemical and thermal-chemical analyses.
Results: Notable lignin modification was achieved from the predominately synergistic down-regulation of S-monomer synthesis in three mutants. This consequently upgraded lignocellulose porosity by up to

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1.8 folds to account for significantly enhanced biomass saccharification and bioethanol production by 20 %-26 % relative to the wild-type. The modified lignin also favors the dissection of diverse lignin nanoparticles with dimensions reduced by 1.5–1.9 folds, applicable for thermal-chemical conversion into the carbon quantum dots with increased yields by 15 % and 31 %. The proportions of G-monomers and FA were significantly increased in the mutants, and the lignin extractions were further assayed with higher activities for two standard antioxidants (DPPH and ABTS) *in vitro* compared to the wild-type, revealing a distinctively enhanced plant antioxidative capacity in the mutants. Water culture showed that young mutant seedlings accumulated more Cd than wild-type did ($p < 0.01$, n = 3), suggesting effective heavy metal phytoremediation in the mutants.

Conclusion: A hypothetical model of characteristic lignin modification for specific S-monomer reduction, accountable for improved lignocellulose recalcitrance, was proposed. It provides a powerful strategy for achieving high-yield biofuels and value-added bioproducts or enhancing plant antioxidative capacity for heavy metal phytoremediation.

Introduction

Lignocellulosic biorefineries are critical for empowering the bioeconomy from advanced biofuels and valuable bioproducts [1]. However, the recalcitrance of lignocellulose fundamentally restrains its enzymatic hydrolysis, leading to costly biomass conversion and secondary waste release into the environment [2–4]. In principle, lignocellulose recalcitrance accounts for plant cell wall composition, polymer feature, and interlinkage [5,6]. In particular, lignin plays a crucial role in recalcitrance from its substantial deposition, which reinforces the impermeable structure of plant cell walls and causes cellulose microfibrils inaccessible for enzymatic digestion. In addition, it impedes lignin exaction and conversion for bioproduction [7,8]. Therefore, genetic and chemical lignocellulose modifications have been integrated to realize low-cost biofuels and high-value bioproducts under green processes [9].

As cellulosic ethanol has been evaluated to be an excellent additive into gasoline, lignin is increasingly being considered a partial substitute for fossil fuels [10,11]. Concerning its chemical properties, lignin is convertible to various bioproducts, and thermal-chemical treatments have been conducted with distinct lignin substrates to generate desirable nanocarbons [12,13]. As it contains several functional groups such as hydroxyl, methoxyl, carbonyl and carboxyl, lignin is progressively used as an effective antioxidant scavenger by reducing oxygen radicals and stabilizing oxidation reactions [14,15]. Although it has significant potential as a sustainable material to produce value-added bioproducts, lignin is particularly recalcitrant to existing chemical technologies, owing to its natural complexity, high degree of polymerization and stable chemical bonds [16,17]. Thus, lignin valorization poses challenges regarding selectivity, yield, and efficiency [18–20]. Similarly, it is technically difficult to generate highly valuable nanomaterials because lignin substrates have large particle size, heterogeneity, poor dispersibility and irregular morphology [21,22]. Nevertheless, genetic engineering of lignin biosynthesis is a promising solution for producing desired lignin substrates convertible for assorted bioproduction.

Lignin is an aromatic heteropolymer consisting mainly of *p*-hydroxyphenyl (H), syringyl (S), and guaiacyl (G) units, which are polymerized by the radical coupling of three monolignols: *p*-coumaryl, sinapyl and coniferyl alcohols [23–25]. Since the major enzymes involved in lignin biosynthetic pathways are identified, genetic engineering of lignin biosynthesis has been attempted to improve lignin recalcitrance in various plants [26–29]. For example, the down regulation of caffeic acid *O*-methyltransferase (COMT), which is involved in monolignol biosynthetic pathways, has led to a remarkable enhancement in biomass enzymatic saccharification [30–35]. Because CRISPR/Cas9 technology is applicable for genome editing in plants, precise mutations of the major genes associated with lignin biosynthesis have been attained in different crops [36]. Despite the structural complexity and biological functions of plant cell walls, precise genetic engineering of lignin biosynthesis may provide valuable information for insights into the specific functions of lignin on biomass conversion or plant antioxidative responses to abiotic stresses.

Rice is a major food crop with large amounts of lignocellulose-rich straws over the world, and also is a desired genetic-model plant for efficient gene-editing [34–37]. In this study, we identified one *OsCOMT* gene preferentially expressed in lignocellulose-rich stem tissue and generated distinct mutants using the CRISPR/Cas9 tool. We then examined the lignin composition and lignocellulose porosity in three independent homozygous mutants (*Oscomts*) and the wild-type (WT), and determined their biomass enzymatic saccharification and bioethanol production under NaOH pretreatment. Chemical and thermal-chemical processes were performed to generate dimension-reduced lignin nanoparticles (LNPs) and carbon quantum dots (CQDs). Notably, we detected a significantly improved antioxidative capacity *in vitro* and efficient cadmium (Cd) accumulation *in vivo* in young seedlings of the mutants. Based on the major findings achieved, this study proposes a mechanism model for characteristic lignin modification by predominately reducing S-monomer synthesis, thereby providing an integrated strategy for precisely genetic-engineering of lignin biosynthesis and efficiently chemical-catalysis of lignocellulose in bioenergy crops.

Results

Selection of oscomt mutants involved in catalyzation of lignin biosynthesis

By searching the TIGR database, this study initially identified DNA sequences matching to the *OsCOMT* family in rice, and generated an unrooted phylogenetic tree from alignments of OsCOMT proteins along the orthologous AtCOMTs in *Arabidopsis* [33–35]. In a comparison, one OsCOMT was closest to the AtCOMT in terms of protein similarity (99 % identity) and motif constitution (Fig. S1), and its *OsCOMT* gene was prominently expressed in the lignocellulose-rich tissues such as the culm, mature sheath, panicle, hull, and spikelet, according to the *OsCOMT* co-expression profiling obtained from the CREP database (https://crep.ncpgr.cn) including the genome-wide expressions of 33 tissues almost throughout the entire life cycle of rice (Fig. S2). This revealed that the *OsCOMT* identified was an appropriate target gene for genomic editing. To perform CRISPR/Cas9 genomic editing, we selected one specific region in exon 3 of the *OsCOMT* gene with a 20 bp target site for designing a sgRNA using the CRISPR-P program. Binary constructs carrying the sgRNA within the target region with Cas9 dri-

ven by UBIp were generated (UBIp: Cas9-OsCOMT), and transformed into the Japonica rice cultivar (Nipponbare/NPB) via agrobacterium-mediated transformation, resulting in 59 independent transgenic lines. A total of 43 positive transgenic lines were thus identified by PCR, and 10 lines were sequenced to verify gene editing. Finally, this study screened out three independent homozygous transgenic *OsCOMT*-edited lines termed as *Oscomt-1,-2,-3,* which exhibited a 20 bp deletion (*Oscomt-1*) and 1 bp addition (*Oscomt-2,-3*) in the target regions for the knock-out of *OsCOMT* in three mutants (Fig. S3).

In the field experiments over six years, we observed slightly affected plant growth and development among the three *Oscomt* mutants, compared with the WT (NPB) (Fig. 1a). Despite the relatively shorter plant heights, the three mutants maintained similar biomass yields to the WT (Table S1), which was partially confirmed by growing one representative mutant (*Oscomt-1*) in two distinct ecological regions on large scale (Table 1). By performing our previously-established chemical analyses with mature rice straws [9,34], we detected significantly reduced lignin levels by 15 %–16 % at $p < 0.01$ (n = 3) in the three mutants (Fig. 1b), but examined relatively higher hemicellulose and cellulose contents by 7 %–14 % as compared with the WT (Fig. S4), indicating that the raised wall polysaccharides may compensate for the reduced lignin deposition to maintain similar biomass yields in three mutants. In addition, similar monosaccharide compositions of hemicelluloses were detected between the three mutants and the WT (Table S2), suggesting that hemicellulose biosynthesis was not affected in the mutants.

Concerning lignin reduction in three mutants, we conducted ^{13}C–^{1}H HSQC 2D NMR analysis to profile the monolignol constitution and chemical linkages of lignin (Fig. 1c; Fig. S5). The S-monomer proportions of the three *Oscomt* mutants were from 21 % to 24 % (of total aromatics), almost 2-fold less than that of the WT (42 %). Accordingly, the three mutants showed higher proportions of the G-monomer (74 %–76 %) and FA (13 %–15 %) than the WT did (53 % and 7 %), whereas their tricin levels were decreased by 53 %–60 % relative to the WT. The FA levels were further confirmed by HPLC analysis (Fig. 1d), which suggested that down-regulation of S-monomer synthesis may occur for the relatively increased G-monomer and FA accumulation in the mutants. Meanwhile, much lower H-monomer proportions were determined in both mutants and WT, consistent with the previous reports [33–35]. Notably, NMR profiling revealed a dual alteration of major monolignol linkages in the three mutants (Fig. S5). Compared to the WT, the mutants showed a unique reduction of β-O-4 proportion, but had a much increase of the β-5 and γ-ester bonds, being agreement with the decrease in lignin biosynthesis in terms of S-monomer suppression. In addition, as the mutants were of increased β-Xyl2 proportion, it is assumed that the down regulation of lignin biosynthesis may relatedly alter lignin interlink with hemicellulose in three mutants.

Enhanced biomass saccharification and bioethanol conversion

Regarding lignin reduction in three mutants, this study examined biomass saccharification by estimating both hexoses and total sugar yields released from the enzymatic hydrolysis of mature rice straws without pretreatment. As a comparison, three mutants showed significantly enhanced biomass enzymatic saccharification compared to the WT at $p < 0.01$ levels (n = 3) with increased rates of 9 %–26 % (Fig. S6). Under our previously established pretreatment with 1 % H_2SO_4 [9], three mutants showed the biomass saccharification increased by 34 %–66 % relative to the WT (Fig. S6). Notably, after mild alkali (1 % NaOH) pretreatment, the three mutants exhibited almost complete cellulose hydrolysis with hexoses yields ranging from 97 % to 100 % (% cellulose). In contrast, the WT had the hexoses yield of 77 % (Fig. 2a & b). By further performing classic yeast fermentation for hexoses conversion into ethanol, this study examined consistently higher bioethanol yields (% dry matter) in three mutants with increased rates of 16 %–32 % ($p < 0.01$, n = 3) compared to the WT (Fig. 2d). Based on the total sugars yield obtained from pretreatment and enzymatic hydrolysis (Fig. 2c), the three mutants could achieve total bioethanol yields of 16 %–17 %, whereas the WT only produced bioethanol of 13 % by calculating xylose-ethanol conversion in theory (Fig. 2e). Because of their significantly reduced lignin levels and relatively increased cellulose and hemicellulose contents, the three mutants could consistently achieve much enhanced biomass enzymatic saccharification for high-yield bioethanol production, even under mild chemical pretreatments.

Effective lignin extraction for upgraded biomass porosity

To understand almost complete biomass enzymatic saccharification under 1 % NaOH pretreatment in three mutants, we examined the wall polymer levels remaining in the pretreated residues (Fig. 3a). Compared to the raw materials (without pretreatment), approximately 53 %–58 % lignin was extracted from alkali pretreatment in the three mutants with 42 % lignin removal in the WT, but both mutants and WT showed similar hemicellulose extraction rates of 6 %–9 % (Fig. 3b; Fig. S7), which is consistent with the altered interlinkages between lignin and hemicelluloses as described above.

Because lignin extraction can improve biomass porosity for enzyme accession and loading [38,39], we performed Simon's staining of lignocellulose substrates after 1 % NaOH pretreatment. Compared with the raw materials, the alkali-pretreated lignocellulose substrates showed significantly increased yellow (DY) and blue (DB) dyes adsorption capacities in both mutants and WT samples (Fig. S8). However, three mutants had much higher DY and DB values than the WT did in the alkali-pretreated lignocellulose substrates (Fig. 3c). Because DY and DB values account for the large and small pore sizes of lignocellulose substrates, respectively [40], this study conducted correlation analyses among wall polymers extraction levels, DY/DB adsorption values and hexoses or total sugars yields. As a result, three parameters (DY, DB, and DY + DB/total pores) of biomass porosity were positively correlated with lignin extraction levels at $p < 0.01$ (n = 3), but not with hemicellulose extraction (Fig. 3d). Notably, the biomass porosity was also significantly positive to account for the hexoses and total sugars yields released from the enzymatic hydrolysis of raw materials and pretreated-lignocellulose substrates in both mutants and WT (Fig. 3e). The lignin extraction rate and level from alkali pretreatment showed a significant positive correlation with hexoses and total sugars yields, whereas no correlation was found with hemicellulose extraction (Fig. S9). Meanwhile, we observed obviously rougher surfaces of the pretreated-lignocellulose substrates in three mutants than the WT under scanning electron microscopy, which was quantitatively confirmed by measuring their surface roughness (Fig. S10). Therefore, the results revealed that effective lignin extraction could play a major role in upgrading biomass porosity for enhanced biomass enzymatic saccharification in the lignin-modified mutants.

Size-reduced lignin nanoparticles and high-yield carbon quantum dots

Because the three mutants had reduced lignin levels and altered monolignol constitution and interlinkage, we measured their molecular weights ranging from 4929 Da to 5415 Da, which were much smaller than those of the WT at 7668 Da (Fig. 4a). Moreover, the three mutants had lower Mw/Mn ratios accounting for their more uniform molecular distributions relative to the WT

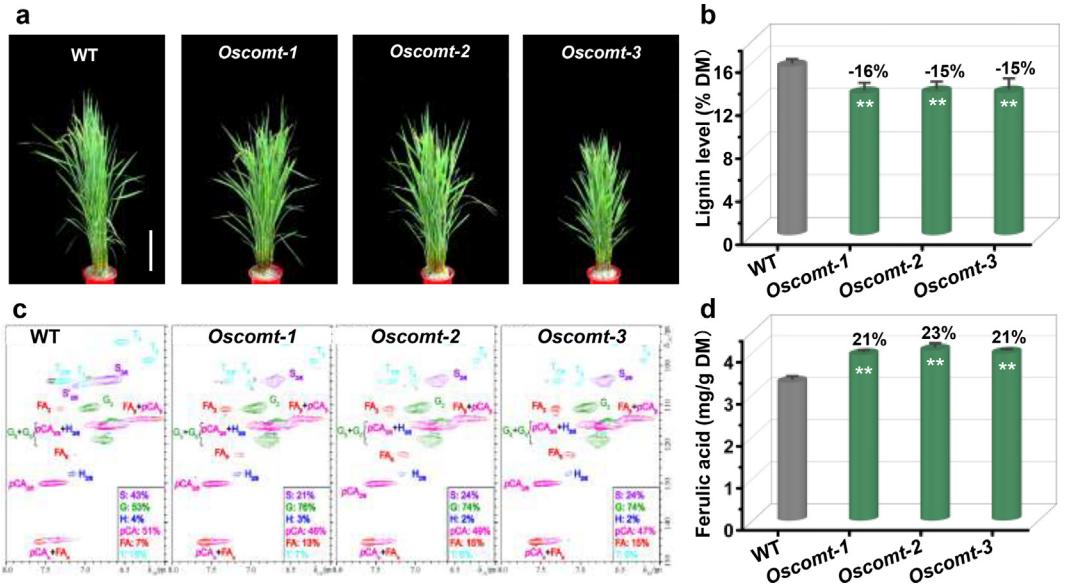

Fig. 1. Characterization of lignin levels and composition in three rice *Oscomt* site-mutants (*Oscomt-1,–2,–3*) and wild type/WT. (a) Plant images at heading stage (scale bar as 15 cm); (b) Lignin level of mature rice straws; (c) 2D HSQC-NMR spectra of lignin composition and linkages; (d) Chemical analysis of ferulic acid. DM: Dry matter, Data as means ± SD (n = 3) with Student's *t*-test as significant differences between mutants and WT at $^{**}p < 0.01$, and the percentage (%) calculated by subtraction between mutant and WT values divided by WT.

Table 1
Agronomic traits of rice *Oscomt-1* mutant & WT grown in Beijing and Wuhan.

Planting region	Sample	Plant height (cm)	Tillering number	Grain yield ($t{\cdot}ha^{-1}$)	Biomass ($t{\cdot}ha^{-1}$)	Harvest index (%)	Panicles m^{-2}	Spikelets Panciles^{-1}	Spikelets $m^{-2}(\times10^3)$	Grain filling (%)	1000-grain weight (g)
Beijing (N40°13′54″, E116°33′50″)[#]	WT	115.3 ± 2.4	11.1 ± 0.4	5.5 ± 0.1	12.4 ± 0.3	38.9 ± 1	274.8 ± 4.5	105.5 ± 4.5	29 ± 1.4	73.3 ± 3.4	23.3 ± 0.3
	Oscomt	98 ± 3.1**	12.4 ± 0.6*	5.6 ± 0.3	11.8 ± 0.7	42.6 ± 0.6*	319.8 ± 10.1**	89.4 ± 5*	28.6 ± 2	77.9 ± 4.2	22.5 ± 0.1
Wuhan (N30°27′55″, E114°21′20″)	WT	120.2 ± 4.1	15.6 ± 0.6	5.3 ± 0.5	13.8 ± 1.1	34.6 ± 0.5	376.2 ± 36.7	92.9 ± 6.9	34.8 ± 2.4	58.7 ± 1.4	23.3 ± 0.3
	Oscomt	101.3 ± 2.7**	15.3 ± 1.1	5.3 ± 0.4	12.5 ± 0.7	37.5 ± 1.1*	407.6 ± 30.4	81.5 ± 2.7	33.2 ± 2.1	64 ± 2.7	22.5 ± 0.1

[#] Latitude and longitude; Data as means ± SD (n = 3) with Student's *t*-test as significant difference between mutant and WT at $^{**}p < 0.01$ or $^{*}p < 0.05$.

(Table S3). Consequently, we performed a simple microwave treatment to generate lignin nanoparticles (LNPs) using total lignin and two other lignin substrates extracted from 1 % NaOH pretreatment and sequential enzymatic saccharification in two representative mutants and WT. Based on dynamic light scattering analysis, largely varied LNP diameters were measured from 10 nm to 396 nm among the three types of lignin substrates; much smaller LNPs (10 nm-14 nm) were generated in the lignin substrates released from the enzymatic hydrolysis of alkali-pretreated lignocelluloses (Fig. 4b–d). In contrast, two representative mutants produced much smaller LNPs than the WT in all lignin substrates, consistent with their reduced lignin molecular weights. Furthermore, we observed the morphological distribution of LNPs from total lignin substrates under transmission electron microscopy, and the LNPs diameters were significantly decreased by 35 %–48 % in the mutants at $p < 0.01$ level compared with the WT (Fig. 4e), demonstrating a remarkable size-reduced LNPs assembly in the mutants. In particularly, one mutant produced significantly homogeneous LNPs than the WT, which was confirmed by the much lower coefficient of variation (CV) of the mutant at 11.3 % relative to the WT at 20.9 %.

To detect the function of the size-reduced LNPs, this study generated carbon quantum dots (CQDs) from 200 °C treatment (Fig. 4f & Fig. S11). Under UV light, blue fluorescence was observed to account for the CQDs intensity, and quantum yields were measured from the fluorescence and UV–vis absorption spectra (Fig. 4g). Remarkably, two representative mutants showed their quantum yields increased by 15 % and 31 % relative to the WT, indicating that genetic engineering of lignin biosynthesis via *OsCOMT*-editing could reduce the lignin level and molecular weight *in vivo*, and cause size-reduced LNPs assembly to produce high-yield CQDs *in vitro*.

Enhanced antioxidant capacity for Cd phytoremediation

As antioxidant activity is a major property of lignin substrates, this study performed two distinct chemical-oxidative assays with total lignin and the residual lignin obtained after 1 % NaOH pretreatments in two representative mutants and WT (Fig. 5a & b). In terms of DPPH radical scavenging activity, a well-defined antioxidant assay *in vitro* [41], we observed that the DPPH solution changed from purple to yellow during incubation with lignin substrates (Fig. 5a), indicating that lignin substrate could capture free radicals from DPPH to account for scavenging activity. Incubated with total lignin substrates, the two representative mutants showed significantly higher scavenging capacities than the WT by 9 % and 10 % at $p < 0.01$ (n = 3). Although alkali pretreatment could cause lignin oxidation for reduced scavenging activity, the residual lignin sub-

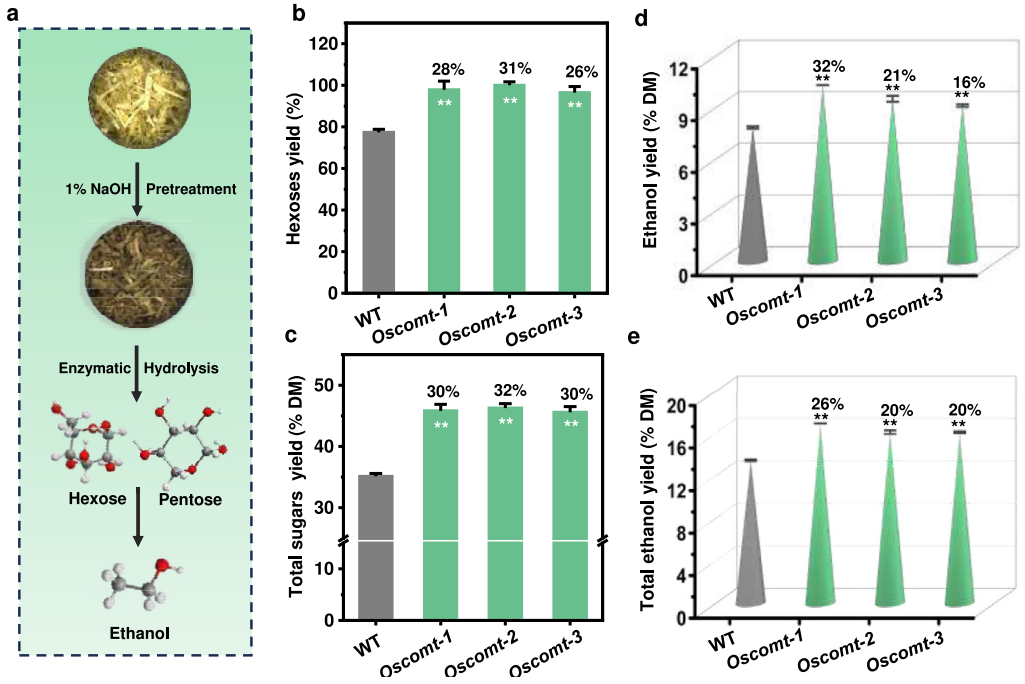

Fig. 2. Biomass enzymatic saccharification for bioethanol production after 1 % NaOH pretreatments with mature rice straws in three *Oscomt* site-mutants and WT. (a) A model for biomass degradation and ethanol fermentation; (b, c) Hexoses and total sugars (hexoses + pentoses) released from enzymatic hydrolysis; (d) Ethanol yield by yeast fermentation; (e) Total ethanol yield calculated in theory from hexoses and xylose co-fermentation. Data as means ± SD (n = 3) with Student's *t*-test as significant differences between mutants and WT at $^{**}p < 0.01$, and the percentage (%) calculated by subtraction between mutant and WT values divided by WT.

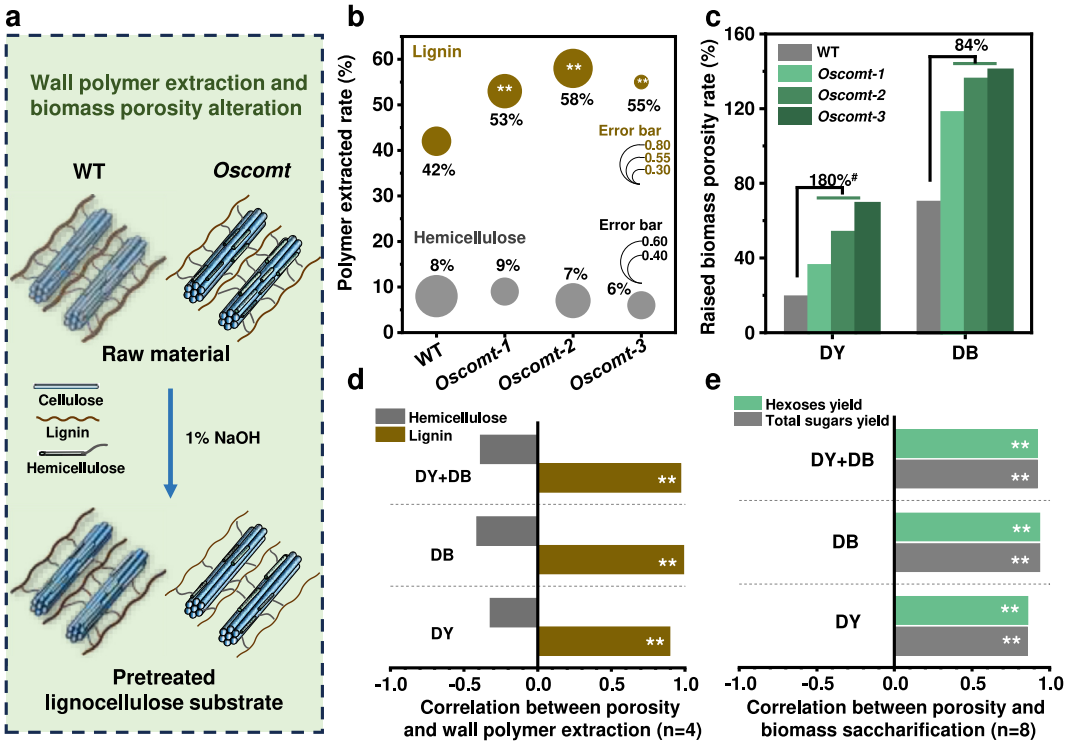

Fig. 3. Characterization of wall polymer extraction and biomass porosity alteration after 1 % NaOH pretreatments with mature straws in three *Oscomt* mutants and WT. (a) A model for wall polymer extraction and biomass porosity augment; (b) Lignin and hemicellulose extraction rates; (c) Raised biomass porosity rates by DY (direct yellow) and DB (direct blue) dye staining; (d, e) Correlation analyses between biomass porosity value and wall polymer level or enzymatic saccharification value in the pretreated lignocellulose; ** As significant correlation at $p < 0.01$ levels; # As the average of three mutants.

strates of the two mutants showed higher scavenging activities than those of the WT by 19 % and 11 %, providing dual evidence of the improved antioxidant properties of lignin substrates in the mutants. We also determined the other antioxidant activities of total lignin and residual lignin substrates by incubation with ABTS, a typical oxidant-chemical (Fig. 5b). Consistently, the two mutants

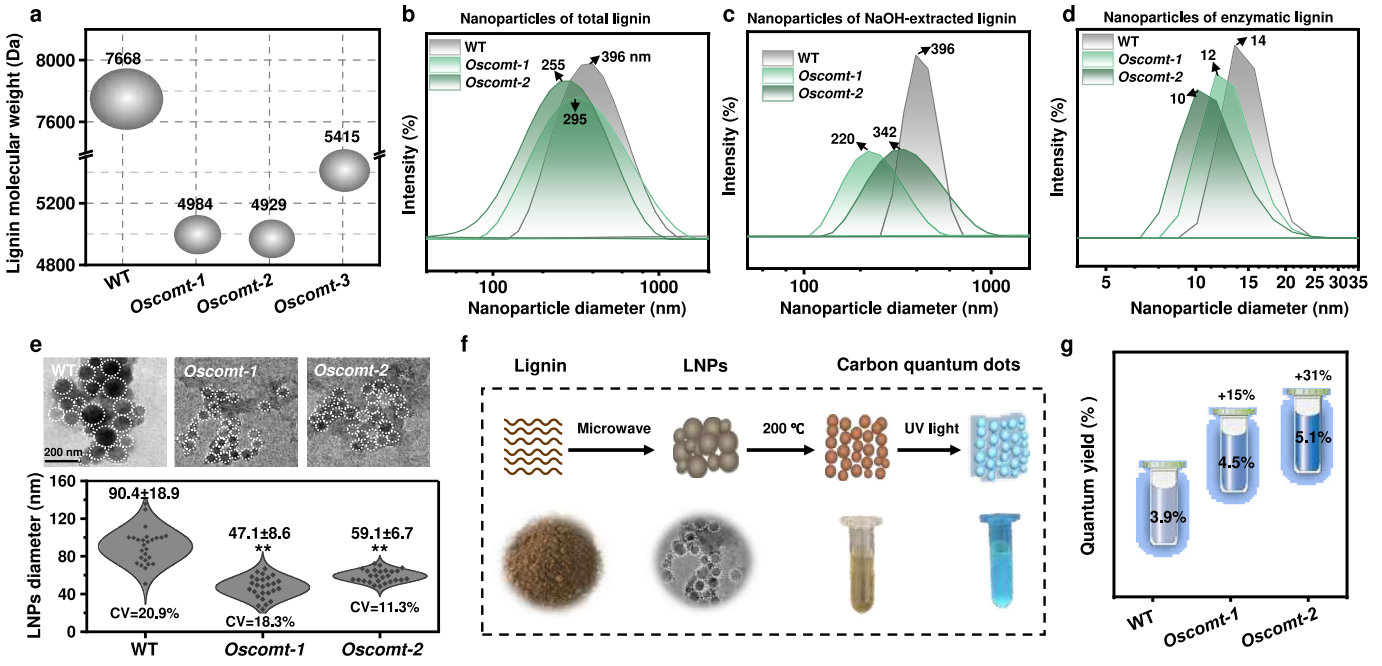

Fig. 4. Lignin-derived nanomaterials generated from *Oscomt* mutants and WT. (a) Molecular weight of lignin; (b) Nanoparticles of total lignin obtained after 67 % H_2SO_4 extraction; (c) Nanoparticles of the lignin substrate extracted with 1 % NaOH pretreatment; (d) Nanoparticle of lignin residues after enzymatic hydrolysis of pretreated lignocellulose substrates; (e) TEM image and diameter of lignin nanoparticles (LNPs), Coefficient of variation (CV), data as means ± SD (n = 25) with Student's *t*-test as significant differences between mutants and WT at $^{**}p < 0.01$; (f) A schematic diagram for CQDs preparation; (g) Quantum yield by ultraviolet excitation.

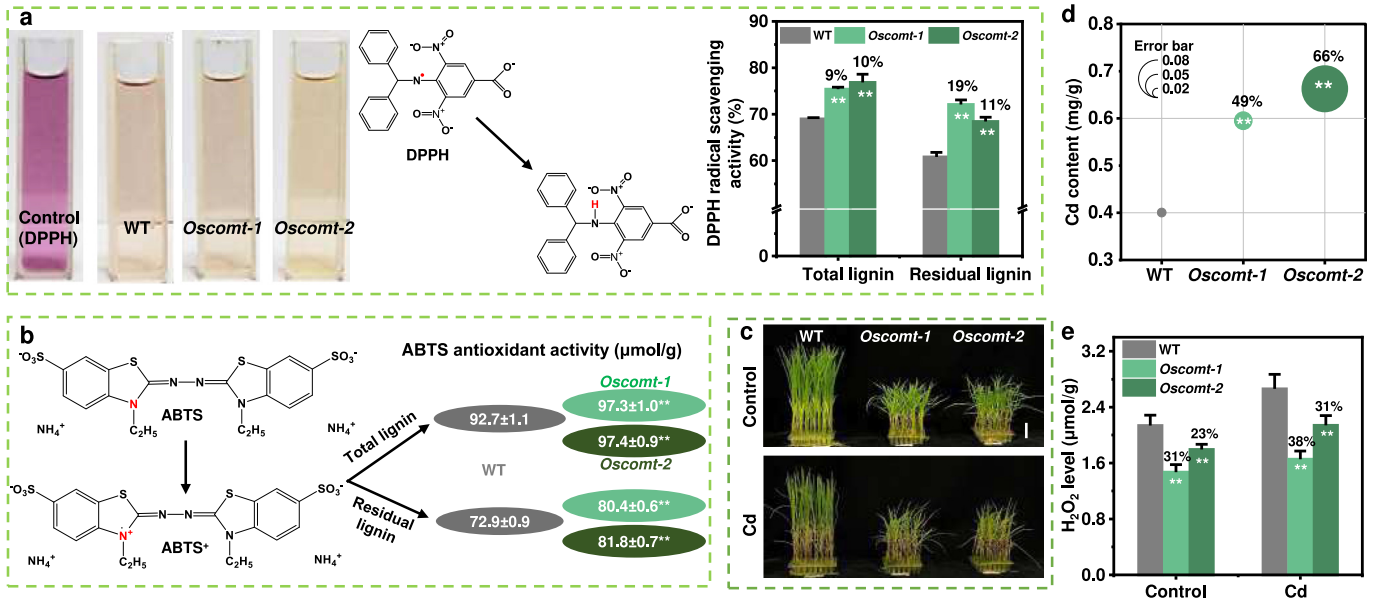

Fig. 5. Characterization of antioxidant activity in *Oscomt* mutants and WT. (a) DPPH radical scavenging by total lignin extracted from raw material and residual lignin obtained after 1 % NaOH pretreatment; (b) ABTS antioxidant activities; (c) The images of rice 30-day-old seedlings co-supplied with 0.2 mM Cd for 15 days; (d, e) Cd and H_2O_2 contents of rice seedlings. Data with error bars as means ± SD (n = 3) with Student's *t*-test as significant differences between mutants and WT at $^{**}p < 0.01$.

showed significantly higher antioxidant activities than those of the WT at $p < 0.01$ levels, which reconfirmed the much-enhanced antioxidant capacities in the mutants.

To determine the biological function of the antioxidant properties of lignin in plant growth and development, we conducted a hydroponic culture with young rice seedlings co-supplied with Cd, a stable oxidant chemical (Fig. 5c). Even though Cd accumulation could cause oxidative damage in plants [42,43], this study determined much higher Cd contents in the young seedlings of two mutants with increased Cd rates by 49 % and 66 % relative to the WT (Fig. 5d). Accordingly, we measured the H_2O_2 levels of young seedlings (Fig. 5e), which has been defined as a parameter directly accountable for the degree of plant oxidation *in vivo* [44,45]. Without any Cd supplementation, the two representative mutants contained significantly lower H_2O_2 levels than the WT by 23 % and 31 % at $p < 0.01$ (n = 3), which was consistent with the much-improved antioxidant capacity of the lignin substrates examined. Under Cd supply, the two mutants and WT showed significantly increased H_2O_2 levels of 13 %, 20 %, and 24 %, respectively (Fig. S12), indicating distinct oxidative responses to Cd stress in these plants. Nevertheless, despite much more Cd accumulation in their young seedlings (Fig. 5d), the two mutants maintained sig-

nificantly lower H_2O_2 levels than those of the WT by 31 % and 38 % (Fig. 5e), indicating the lignin modification of *Oscomt* mutants could remarkably improve plant antioxidant capacity for enhanced Cd phytoremediation.

Discussion

Plant cell walls represent substantial biomass resources transformable into renewable biofuels and valuable bioproducts; however, lignocellulose recalcitrance primarily determines costly biomass processes [46]. Considering that lignin is a major contributor to the recalcitrance, genetic engineering of lignin biosynthesis has been attempted to reduce lignin deposition along with the modification of lignin-carbohydrate complexes in various bioenergy crops [8,9,38]. As lignin biosynthesis requires several enzymes for multistep catalysis [47,48], the selection of an appropriate gene for effective genetic-manipulation is increasingly being considered a primary effort for lignin modification. Based on major findings as previously achieved [43,44], this study proposes a hypothetical model of lignin modification by highlighting the potential silencing frequences of COMTs in the *Oscomt* mutants (Fig. 6a). As the COMTs have isoforms for putative catalytic activities [49,50], this study attempts to demonstrate that genetic knock-out of *OsCOMT* gene could cause a predominately reduced S-monomer synthesis for relatively increased G-monomer and FA proportions examined in the three mutants (Fig. 1). However, such hypothesis requires a further clarification via biochemical assays in the future. Given that COMT is directly involved in catalysis of FA, G- and S-monomer syntheses, it is assumed that *OsCOMT* could be one of the desirable genes for specific genetic modification of lignin composition and assembly. Conversely, despite three *Oscomt-1,2,3* mutants show different mutation sites for genomic knock-out of *OsCOMT*, they all remain similar in plant growth phenotype and lignin biosynthesis regulation, indicating that genome editing with *OsCOMT* via modern CRISPR/cas9 technology should be effective for the promising genetic-modification of lignin biosynthesis in bioenergy crops.

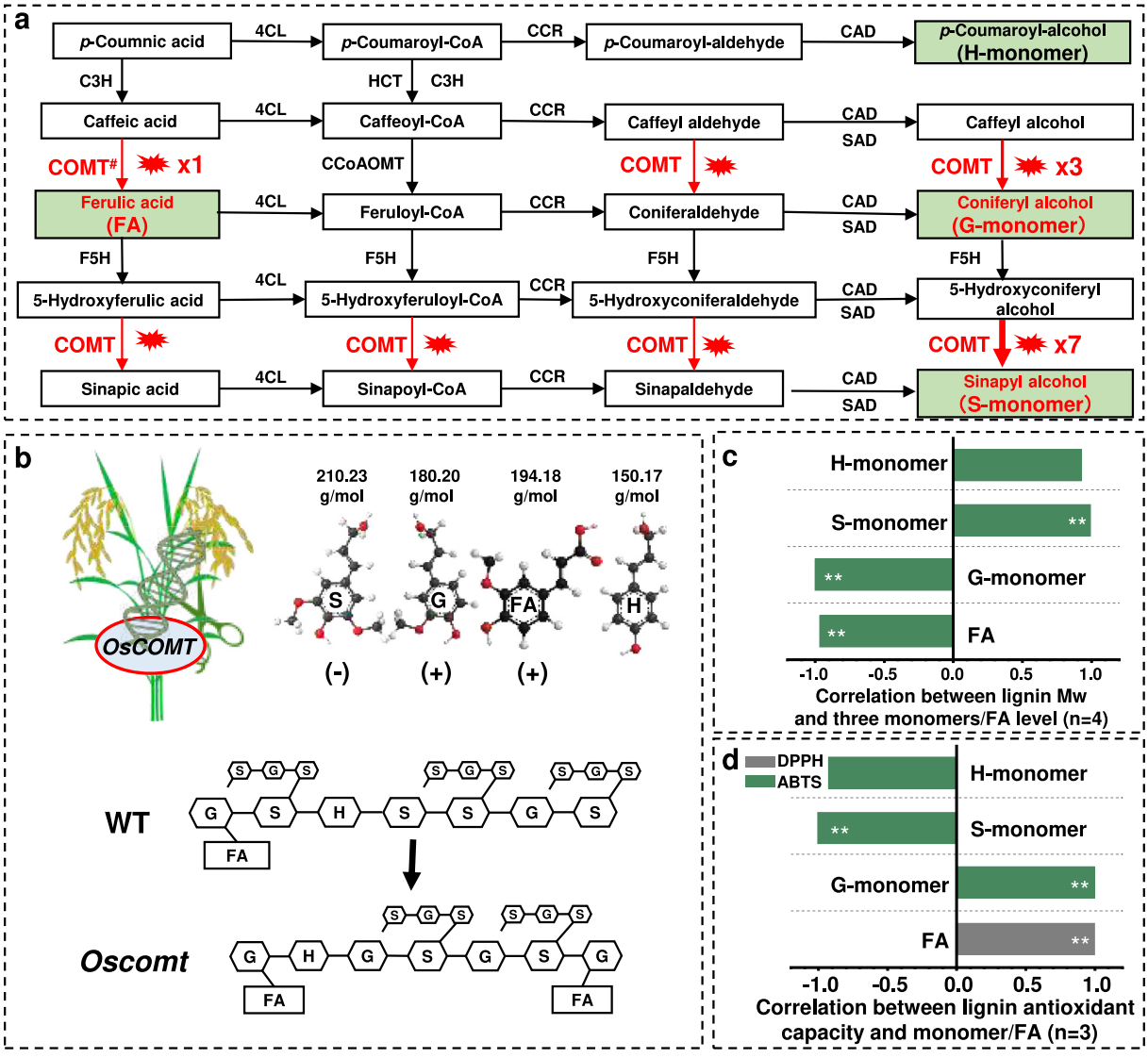

Fig. 6. Characterization of distinct OsCOMT inhibitions of three monomer (H, G, S) and FA syntheses accountable for characteristic lignin assembly *in vivo* and functional nanomaterial generation *in vitro* in *Oscomt* mutants. (a) Three-monomer and FA synthetic pathways, red sparking as inhibition in site mutant, # As COMT was repressed with one site (x1); (b) *OsCOMT* site mutations for lignin modification; Correlation analyses between three monomers/FA levels and lignin Mws (c) or lignin antioxidation activity (d); * and ** as significant correlations at $p < 0.05$ and 0.01 levels. 4CL 4-coumarate CoA ligase, C3H 4-coumarate 3-hydroxylase, F5H Ferulate 5-hydroxylase, CCR Cinnamoyl-CoA reductase, HCT Quinate shikimate *p*-hydroxycinnamoyl transferase, CAD Cinnamyl alcohol dehydrogenase, COMT Caffeic acid O-methyltransferase, CCoAOMT, Caffeoyl-CoA O-methyltransferase, SAD Sinapyl alcohol dehydrogenase.

As the S-monomer has a relatively higher molecular mass than the G-monomer and FA compounds, the significantly decreased lignin levels and molecular weights in the three mutants could be mainly due to their predominantly reduced S-monomer levels and relatively increased G-monomer and FA contents (Fig. 6b). These findings are confirmed by either the negative correlation between lignin molecular weight and S-monomer level or the positive correlation between lignin molecular weight and G-monomer/FA content at $p < 0.01$ among the three mutants and wild-type samples examined (Fig. 6c). Given that genetic engineering of lignin biosynthesis has, in principle, focused on reducing lignin deposition for improved lignocellulose recalcitrance in bioenergy crops [2,26], this study achieves an effective lignin extraction from mild alkali pretreatment for remarkably increased biomass porosity in three mutants, which should be mainly subjective to their reduced lignin molecular weight and altered lignin composition and interlink with xylan. Importantly, such effective lignin extraction causes an almost complete biomass enzymatic saccharification towards higher bioethanol production and enables the generation of different types of size-reduced LNPs with higher homogeneity, which should be the major cause for the high yield of CQDs in the mutants.

Because plant cell walls consist of complicated structures with diverse biological functions [2,51,52], genetic modification of lignocellulose can simply cause defects in plant growth and adaptation to environmental stresses. Although the three *Oscomt* mutants had significantly reduced lignin levels and altered monolignol composition, they all exhibited slightly affected plant growth and biomass yields, mainly because of the significantly increased hemicellulose and cellulose contents (Fig. S4), suggesting that CRISPR/cas9 editing of *OsCOMT* may also indirectly regulate the biosynthesis of other cell wall polysaccharides in secondary cell walls. Thus, it differs from most of the previously identified mutants with defective phenotypes [53,54]. Furthermore, the hypothetical model indicated that relatively increased G-monomer and FA contents and a significantly reduced S-monomer level may cause a synergistic enhancement of lignin antioxidant capacity *in vivo*, owing to their significantly positive correlations with the two standard antioxidants *in vitro* (Fig. 6d). Because Cd accumulation can drastically cause oxidative stress in plants [6,42], the hypothetic model should explain why the three mutants could accumulate much more Cd for phytoremediation. As plant antioxidative capacity is defined as an integrated parameter accounting for plant responses to various environmental stresses [55–57], it would be interesting to test the resistance and tolerance of the three mutants to other biotic and abiotic stresses in the field experiments.

In conclusion, this study demonstrates that CRISPR/cas9 editing with *OsCOMT* can distinctively improve lignocellulose recalcitrance by synergistic down-regulation of S-monomer synthesis. This could greatly enhance biomass enzymatic saccharification for high-yield bioethanol production, and consequently generate side-reduced LNPs for yield-raised CQDs. Notably, it is also found that specific engineering of lignin biosynthesis has little impact on plant growth, but significantly increases plant antioxidant capacity for Cd phytoremediation. Therefore, this study provides a powerful strategy for the optimal genetic-modification of lignocellulose substrates for high-yield biofuels and value-added bioproducts along with enhanced plant antioxidation capacity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2025.01.048.

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