Enhancing digestibility and ethanol yield of *Populus* wood via expression of an engineered monolignol 4-O-methyltransferase

Yuanheng Cai¹*, Kewei Zhang¹, K. Hoon Kim², Guichuan Hou³, Xuebin Zhang¹, Huijun Yang¹, Huan Feng¹, Lisa Miller⁵, John Ralph² & Chang-Jun Liu¹

Producing cellulosic biofuels and bio-based chemicals from woody biomass is impeded by the presence of lignin polymer in the plant cell wall. Manipulating the monolignol biosynthetic pathway offers a promising approach to improved processability, but often impairs plant growth and development. Here, we show that expressing an engineered 4-O-methyltransferase that chemically modifies the phenolic moiety of lignin monomeric precursors, thus preventing their incorporation into the lignin polymer, substantially alters hybrid aspens' lignin content and structure. Woody biomass derived from the transgenic aspens shows a 62% increase in the release of simple sugars and up to a 49% increase in the yield of ethanol when the woody biomass is subjected to enzymatic digestion and yeast-mediated fermentation. Moreover, the cell wall structural changes do not affect growth and biomass production of the trees. Our study provides a useful strategy for tailoring woody biomass for bio-based applications.

¹ Biology Department, Brookhaven National Laboratory, Upton, New York 11973, USA. ² Department of Biochemistry, and the DOE Great Lakes Bioenergy Research Center, The Wisconsin Energy Institute, University of Wisconsin, Madison, Wisconsin 53726, USA. ³ Dewel Microscopy Facility, Appalachian State University, Boone, North Carolina 28608-2027, USA. ⁴ Department of Earth and Environmental Studies, Montclair State University, Montclair, New Jersey 07043, USA. ⁵ National Synchrotron Light Source, Brookhaven National Laboratory, Upton, New York 11973, USA. * These authors contributed equally to this work. † Present addresses: College of Chemistry and Life Sciences, Zhejiang Normal University, Zhejiang 321004, China (K.Z.); Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, New York 14853-4203, USA, (H.Y.). Correspondence and requests for materials should be addressed to C.-J.L. (email: cliu@bnl.gov).
Wood is one of the world’s most abundant natural materials. It is widely used in pulp and paper manufacturing and recently has been considered a promising source of simple sugars for industrial bio-based products and advanced biofuels1. Wood is the secondary xylem of vascular plants, consisting of a compound middle lamella and secondary wall layers composed of a cellulose/hemicellulose network, and impregnated with lignin. In the wood of *Populus* spp., these polymers occur in the approximate proportions of 45% cellulose, 25% hemicelluloses and 20% lignin2. Lignin in the wood forms a hydrophobic barrier hindering the access of digestive enzymes to polysaccharides and decreasing their activity; therefore, it is a significant limiting factor in converting woody biomass to fermentable sugars in processes towards biofuels3. Lignins are complex and heterogeneous polymers of 4-hydroxyphenylpropanoid units4. They are derived from the oxidative radical coupling of three typical building blocks (that is, the monolignols), p-coumaryl, coniferyl and sinapyl alcohols (Fig. 1a). After their incorporation into the growing lignin polymer, they are oxidatively coupled into the growing polymer forms lignins3,22 (Fig. 1b). In this process, a free phenol (the unsubstituted 4-hydroxy group of a monolignol, and/or the free-phenolic end of the growing polymer), is essential for generating phenolic radicals to allow radical coupling that forms the different types of lignin inter-unit linkages3,13. Previously, we demonstrated that chemical modification, that is, the methylation of the 4-hydroxy group of a monolignol, prevents the participation of the monolignol derivative in the subsequent coupling process, thus disrupting lignin formation23. Based on this observation, we engineered a set of novel *O*-methyltransferase (OMT) variants using structure-guided iterative saturation mutagenesis. These OMT variants effectively catalyse the 4-O-methylation of monolignols, and are therefore termed *monolignol-*4-O-methyltransferases (MOMTs)24 (Fig. 1c). One of these variants, MOMT4, shows a slight catalytic preference for methylating sinapyl alcohol, the *S*-lignin precursor, *in vitro*25. To evaluate its potential effects on lignin synthesis in woody species, and its application in tailoring wood digestibility for biofuel production, in this study we express MOMT4 in hybrid aspen (*Populus tremula × Populus alba*), a fast growing *Populus* species. We demonstrate that the expression of MOMT4 in aspen markedly alters lignin structure, with more than a 50% reduction of the labile S-units and about a fourfold increase in condensed lignin subunit cross-linkages by G-units. However, in contrast to our common perception, along with the increase of condensed lignin structures, the efficiency of the release of simple sugars from transgenic woods increases up to 62%, and the ethanol yield from pretreated and non-treated transgenic woods rise by >40% and 49%, respectively, compared with those from their corresponding controls. Furthermore, the content and composition changes in lignin do not affect growth and fitness of the transgenic aspens in the greenhouse; the wood densities and the biomass yields are comparable to those of the controls. These data suggest that (1) lignin condensation itself is not a critical factor affecting the digestibility of the cell wall; (2) the developed strategy of etherifying lignin precursors by the engineered OMT represents a useful biotechnological solution for effectively tailoring the digestibility of *Populus* woody biomass and, (3) the created transgenic aspens are a promising advanced biomass feedstock for biofuel production.

**Results**

**Alteration of lignin content and structure.** MOMT4, one of engineered enzyme variants from the parent enzyme isoeugenol 4-O-methyltransferase26, possesses four amino acid substitutions in its active site, that is, T133L, E165I, F175I and H169F (ref. 25). Those substitutions confer a substantial catalytic efficiency of the enzyme to both coniferyl alcohol and sinapyl alcohol for *p*-hydroxy methylation. Kinetically, MOMT4 slightly prefers sinapyl alcohol with a catalytic efficiency (*k*<sub>cat</sub>/*K*<sub>m</sub>) of 3,999.3 (M<sup>−1</sup>s<sup>−1</sup>), and a binding affinity (*K*<sub>m</sub>) of 68.1 ± 11.1 (± indicates s.d., *n* = 3) μM versus 2,738.7 (M<sup>−1</sup> s<sup>−1</sup>) and 192.6 ± 24.5 (± indicates s.d., *n* = 3) μM for coniferyl alcohol. We transferred the expression cassette of the MOMT4 gene, driven by the promoter of the bean phenylalanine ammonium lyase-2 (PAL2) gene that encodes the enzyme catalysing the first step of phenylpropanoid/lignin biosynthesis27, into hybrid aspen via *Agrobacterium*-mediated transformation (Fig. 2a). Bean PAL2 promoter has been demonstrated to be highly active in the early stage of vascular development at the inception of xylem differentiation, when it drives the glucuronidase reporter gene, and is heterologously
expressed in tobacco; its promoter property was well conserved, even expressing in gymnosperm species. Furthermore, this promoter has been successfully adopted in driving the target genes to effectively disturb lignin biosynthesis in different plants. After obtaining the independent primary transformants, ~2-month-old transgenic trees grown in a greenhouse were initially screened. Profiling the methanolic extracts from the plants revealed that two novel metabolites had accumulated in the leaves of the established transgenic lines (Supplementary Fig. 1a,b). After digestion of the leaf extract with β-glucosidase, we found that one metabolite we resolved was identical to the 4-O-methylated coniferyl alcohol, and the other one was identical to the 4-O-methylated sinapyl alcohol, based on their retention times and ultraviolet spectra, compared with the enzymatically synthesized authentic standards, as was their molecular mass assignment (detected by gas chromatography–mass spectrometry (GC–MS)) (Supplementary Fig. 1a–f). These data indicate that the engineered MOMT4 functions properly in the transgenic aspens, and can modify monolignols into the corresponding 4-O-methylated compounds; those methylated products most likely were further transformed to their glucoconjugates for detoxification and/or for the storage. The levels of the accumulated metabolites in the leaves of ~2-month-old trees were ~2–3 μmol g⁻¹ of the fresh weight of the leaves for the 4-O-methylated coniferyl alcohol, and 5–7 μmol g⁻¹ fresh weigh for the 4-O-methylated sinapyl alcohol (Supplementary Fig. 1g). Interestingly, in the stems of ~2-month-old trees, only 4-O-methylated sinapyl alcohol was detected, and its accumulation level was much lower than that in the leaves at ~0.12–0.2 μmol g⁻¹ fresh weight, which probably reflects the limited storage capacity of xylem cells for the soluble phenolics. Based on the phenolic profiling and the preliminary quantification of the total lignin content of these generated transgenic lines, we selected four independent lines, covering the lowest- to the highest-level of metabolic changes, for our further detailed histochemical and chemical analyses on the composition of their cell walls. The conventional phloroglucinol-HCl stain that yields a violet-red colour indicative of total lignin revealed that the xylem tissues of MOMT4 transgenic aspens exhibited a slightly weaker (or nearly indiscernible change in) colour intensity than those of the control plants (Fig. 2b,c), suggesting a limited reduction of total lignin content in the wood; however, when we applied the Mäule stain (which stains syringyl units in lignin red), the colour intensity apparent on the cross-section of MOMT4 transgenic stem was undoubtedly fainter than that displayed in the control stem (Fig. 2d,e), indicating a substantial

Figure 1 | Illustration of MOMT4-mediated depression on lignin polymerization. (a) The conventional monolignol structures. (b) Scheme for monolignol oxidative dehydrogenation and polymerization processes. (c) The reaction catalysed by the engineered monolignol 4-O-methyltransferase, which deprives the propensity of the modified lignin precursors for further polymerization.
reduction of S-type lignin subunits in the cell walls of MOMT4 transgenic lines. Consistent with the histochemical observation, quantification of acetyl bromide lignin in the stem cell walls of ~6-month-old plants revealed that the average lignin content of control lines was ~23% of tissue dry weight, whereas of the MOMT4 transgenic lines ranged from 19 to 20%, indicating a moderate level of lignin reduction (Fig. 2f). When the monomeric composition of lignin was determined using the diagnostic thioacidolysis method that primarily cleaves β-O-4-ether linkages interconnecting the lignin subunits [23, 24], we found that the amount of released S monomers from MOMT4 overexpression lines exhibited 63–74% reduction compared with the control lines; in contrast, the amount of released G-units was very similar, with an approximate level of 400 µmol g⁻¹ dried cell wall residues (Fig. 2g). Correspondingly, the S/G ratio of lignin had fallen from a value of 2:1 in the controls to 1:2 in the MOMT4 overexpression lines (Fig. 2g). These data suggest that expression of MOMT4 in hybrid aspen substantially alters lignin composition, and that the incorporation of the S-lignin monomer (sinapyl alcohol) was principally impaired in the cell walls of MOMT4 transgenics.

Two-dimensional heteronuclear single-quantum coherence (2D HSQC) NMR experiments further verified the reduction of S-units in lignins from MOMT4 transgenic lines. The detected signals from the S-units of two independent MOMT4 transgenic lines exhibited >50% depletion over than those of the control lines; the calculated S/G ratio changed from 1.7 in the control lines to 0.4 in the MOMT4 overexpression aspens (Fig. 3a,b; Supplementary Fig. 2), consistent with the thioacidolysis results. In both the thioacidolytic and NMR analyses, the detected amount of the p-hydroxyphenyl (H) units was extremely low, and was not significantly different between the MOMT4 overexpression and control lines (Figs 2 and 3).

The aliphatic region of 2D HSQC NMR spectra reflects the changes in the types and distribution of inter-unit linkages present in the lignin fraction. The lignin from the control lines was rich in β-aryl ether units with modest amounts of resinols and low amounts of phenylcoumarans, as well as modest levels of the cinnamyl alcohol end groups (Fig. 3c; Supplementary Fig. 3). Resinols (β-β-coupled units) mostly arise from the dimerization of sinapyl alcohol. The spectra of lignin derived from MOMT4 transgenic aspens revealed that the resinol and β-aryl ether units were decreased by 25% compared with the controls, consistent with the lower amount of syringyl monomers released by thioacidolysis and documented in 2D HSQC NMR spectra. However, the phenylcoumaran (β-5-coupled) structures that require at least one guaiacyl unit for their formation increased on average by more than fourfold over the controls (Fig. 3d; Supplementary Fig. 3). The level of cinnamyl alcohol end groups that also primarily arise from coniferyl alcohol (G-unit) monomer–monomer coupling [25], rose about 1.5-fold (Fig. 3d; Supplementary Fig. 3). These data affirm that lignin polymer from MOMT4 transgenic aspens is richer in the condensed linkages (for example, β-5 structures), and may be more branched or simply have shorter chains, as hinted by its increased end group units. Indeed, gel permeation chromatography revealed that the distribution of molecule weight (Mₙ) in the milled wood lignin fraction is lower in the

Figure 2 | The effect of expression of MOMT4 on content and composition of lignin in transgenic aspens. (a) Three-month-old hybrid aspens of control (left) and three MOMT4 independent transgenic lines (right). (b,c) Phloroglucinol-HCl staining of the stem cross-sections of control (b) and MOMT4-0 transgenic line (c). (d,e) Mäule staining of the stem cross-sections of control (d) and MOMT4-0 transgenic (e). Scale bars, 1 mm. (f) Acetyl bromide total lignin content in the cell walls of control and MOMT4 transgenic aspen stems. (g) The monomers released by thioacidolysis from the stem cell walls of MOMT4 transgenic aspens; S, syringyl; G, guaiacyl; H, p-hydroxyphenyl; CWR, cell wall residues; Ctrl., control. Data in f,g represent mean ± s.e. with three biological replicates (each with three technical repeats) for the control and three technical repeats for the individual transgenic lines. ** Indicates significant difference of lignin content (f) or S-monomer (g) compared to the control with P<0.01 (Student’s t-test).
MOMT4 transgenics compared with the control samples (Fig. 4), suggesting a lower degree of polymerization.

Alteration of wall-bound phenolics. Hydroxycinnamates (for example, p-coumarate and ferulate) and hydroxybenzoates that bond with the cell walls' non-cellulosic polysaccharides and lignin via ester linkages can act as crosslinkers or play a role in altering the oxidative potential of monolignols. In poplar cell walls, the prominent 'wall-bound' phenolic is p-hydroxybenzoate that predominantly acylates syringyl units. Examining the wall-bound phenolics from the stems of MOMT4 transgenics revealed that the levels of both p-hydroxybenzoate and p-coumarate showed about a 50% reduction, whereas the average amount of ferulate essentially was unchanged, compared with those in the control lines (Fig. 5). Both p-hydroxybenzoate and p-coumarate were proposed to conjugate with sinapyl alcohol within the cell and then incorporate into lignin polymer. The reduction of both p-hydroxybenzoate and p-coumarate esters was consistent with the decreased incorporation of S-lignin monomers.

Alteration of cell wall polysaccharides. Along with changes of lignin content and structure of MOMT4 transgenic aspens, the total cellulose content was found to have increased from 51% of the dried cell wall residues in the controls, to a maximum of 57% in the MOMT4 overexpression lines, that is, by up to a 12%
increase (Fig. 6a). Synchrotron FT-IR imaging further verified that the polysaccharide level in the developing xylem of the stem cross-section of MOMT4 transgenics was substantially higher than that of the controls, whereas the level of lignification in the same developing wood tissues was lower (Supplementary Fig. 4). Along with the increase in total cellulose content, the levels of crystalline cellulose in the walls of MOMT4 transgenics was also increased by up to 15% over the levels of the controls (Fig. 6b), suggesting an ultrastructural change of cellulose fibres. When hemicelluloses were quantified, we found that their abundance in the cell walls was essentially unchanged, accounting for about 22.6% of the cell wall dry mass in the control, and an average 23.2% (range 20.7–25.5%) in the transgenic lines (Fig. 6c). The detected amount of hemicellulosic monomeric sugars also remained essentially the same in most lines, except the glucose content, which may have arisen from the amorphous regions of cellulose fibres, which was slightly higher in one of the transgenics (Supplementary Table 1). These data demonstrate that the alteration of lignin content and structure in MOMT4-overexpressing aspens affects the accumulation and ultrastructure of cellulose fibres, whereas it has less effect on the deposition of hemicelluloses.

**Enhanced cellulytic efficiency and ethanol yield.** When the prepared wood cell walls were treated with a cellulase mixture, all of the examined MOMT4 transgenic samples displayed 50%–62% more weight loss than the controls, indicative of the release of more simple sugars and a higher degree of digestibility (Fig. 7a). Subsequently, two sets of the transgenic and control woods, one from 6-month-old newly propagated plantlets and another from ~1-year-old basal stem grown from the trees after three coppice events, underwent 'simultaneous saccharification and fermentation' to measure ethanol production. Both the MOMT4 transgenic woods and control ones either were treated with alkali (Supplementary Fig. 5a) or left untreated; then after washing the pretreated samples with water, the woody powders were incubated with polysaccharidase enzymes and yeast cells in the fermentation broth. During incubation, we measured the weight loss caused by the release of fermentation gases and the ethanol content in the broth. The data showed that both the pretreated and non-pretreated transgenic woods displayed both a faster and higher fermentative weight loss (that is, the gas release), indicating a higher rate of saccharification and bioconversion compared to the control woods (Fig. 7b; Supplementary Fig. 5b). Correspondingly, higher yields of ethanol were realized compared to their respective controls (Fig. 7c; Supplementary Fig. 5c). When simultaneous saccharification and fermentation proceeded for 168 h, the alkaline-pretreated and non-pretreated wood samples from 6-month-old MOMT4 transgenic plantlets showed up to 24% and 49% increase in ethanol yield, respectively (Fig. 7c). For alkaline-pretreated and non-pretreated wood samples from ~1-year-old basal stem of the primary transgenic plants, ethanol yield increased up to 40% and 48%, respectively,
compared with the controls (Supplementary Fig. 5c). These data indicate that even though the MOMT4 transgenic woods contain a relatively higher level of condensed lignin, their digestibilities and bioconversion rates were substantially improved.

Growth and wood anatomy of transgenic aspens. Although the MOMT4 transgenic aspens exhibited substantial changes in both lignin and cellulose contents and structures, they exhibited normal growth and development in the greenhouse (Fig. 8). The average stem heights, basal stem thicknesses and total dried biomass yields of 3- and/or 6-month-old transgenic trees were statistically similar to those of the controls, notwithstanding the substantial variation among individual transgenic and control plants (Fig. 8a; Supplementary Table 2). The measured wood density of a set of primary transgenic lines, on average, was the same as in the control lines (Fig. 8b). At the cellular level, MOMT4 transgenic aspens developed a normal vasculature, and no collapsed vessel or fibre cells was observed, except that the lumina of their fibres appeared slightly narrower than those of the control line (Fig. 8c,d). These data suggest that expression of MOMT4 and the alteration of the composition and structure of lignin do not significantly compromise the aspen trees’ growth or their production of woody biomass.

Syringyl lignin related gene expression and enzyme activity. Sinapyl alcohol biosynthesis branches from coniferyl alcohol/ aldehyde biosynthesis in the pathway by the sequential catalyses of coniferaldehyde/ferulate 5-hydroxylase (Cald5H/F5H) and COMT. We hypothesized that the reduced S-lignin content in the transgenics might have resulted from the potential inhibitory effect of 4-0-methylated monolignols, the products of MOMT4, on the activities of the biosynthetic enzymes specific for syringyl monomers. We therefore conducted substrate-inhibition assays using the prepared 4-0-methylated coniferyl and sinapyl alcohols on the recombinant aspen enzymes, Cald5H/F5H, COMT, CAD ((hydroxy)cinnamyl alcohol dehydrogenase), and a homologue of the previously identified poplar SAD (sinapyl alcohol dehydrogenase)34, as well as on the crude enzyme extracts (for COMT, CAD/SAD, and peroxidase activities). Except for the recombinant CAD and the SAD homologue 34, where their activities showed a 3–14% inhibition when incubated with 4-O-methylated monolignols (Supplementary Table 3), there was no obvious inhibition on the activities of other recombinant enzymes (Supplementary Table 3). Consistent with a previous study34, the 4-O-methylated monolignols did not impair the peroxidase-mediated oxidative polymerization process of the conventional monolignols (Supplementary Fig. 6).

Figure 6 | The effect of expression of MOMT4 on accumulation of carbohydrates in transgenic aspens. (a) Cellulose content in the cell walls of control and MOMT4 transgenic aspens. (b) The crystalline cellulose content in MOMT4 transgenic cell walls. (c) Total hemicelluloses content in the cell walls of control and MOMT4 transgenic aspens. CWR, cell wall residues; Ctrl., Control. Data in a,b represent mean ± s.d. from three biological replicates (each with eight technical repeats) for the control set and eight technical repeats for MOMT4 OE lines. **P<0.01 (Student’s t-test). Data in c represent mean ± s.e. with three biological replicates (each with three technical repeats) for the control and three technical repeats for transgenic lines. No statistic difference between the transgenic and the control line was detected.
Considering the potential global transcriptional effect of the expression of \textit{MOMT4} on the cell wall and the lignin biosynthetic genes, we conducted a transcriptomic analysis on an \textit{MOMT4} overexpression line using RNA-seq. The data indicated that although the transcripts of \textit{MOMT4} transgene were highly abundant, which verifies the overexpression of the \textit{MOMT4} gene and demonstrates the reliability of RNA-seq dataset, none of the lignin biosynthetic genes exhibited substantial changes in their transcript abundances compared with those of the control set.

Quantitative RT-PCR analysis on a set of xylem-specific lignin biosynthetic genes\textsuperscript{35} further validated that the transcripts of monolignol-branch genes, particularly the S-lignin synthetic genes, \textit{Cald5H/F5H}, \textit{COMT}, \textit{CAD} and \textit{SAD} homologue, did not exhibit significant differences between \textit{MOMT4} transgenic and control lines, although transcripts of a few genes in individual control or \textit{MOMT4} transgenic lines appeared to be outliers (Supplementary Fig. 7). Interestingly, RNA-seq data indicated that, among a small set of genes showing an altered transcript abundance, a few are annotated to encode cell wall proteins, including an \alpha-expansin family of proteins that are potentially involved in cell wall loosening\textsuperscript{36}, a glycosyl hydrolase (9B13) whose homologous members in the family of \textit{endo-1,4-}\beta-glucanases were reported to participate in cellulose synthesis or assembly\textsuperscript{37}, and the cell wall-localized proline-rich protein (Supplementary Table 4). These data suggest the potential involvement of those proteins/enzymes in the observed alteration of the cell wall structure and composition of the \textit{MOMT4} transgenic aspens.

**Discussion**

The production of biofuels and bio-based chemicals from woody biomass is limited by its recalcitrance to hydrolysis for releasing simple sugars. Most lignin elimination processes are inefficient and expensive for many applications. Reducing and/or structurally changing lignin by manipulating certain genes in the monolignol biosynthetic pathway can enhance the cell wall degradability\textsuperscript{1}; however, this approach is often accompanied by impairment of plant growth and development that results in a severe penalty to biomass yield\textsuperscript{6,12,13,17,18}. Plants seem, however, to be tolerant of a wide range of lignin compositional/structural changes. One measure of lignin structure is its G- and S-lignin unit composition. In general, a high S/G ratio is associated with increased pulping yields\textsuperscript{38,39}, or improved enzymatic release of sugars\textsuperscript{40}. The present study demonstrated that expression of the \textit{MOMT4} enzyme that etherifies the phenolic moieties of monolignols preferentially disrupts S-lignin deposition in hybrid aspen, which leads to the production of a G-lignin-rich biopolymer. An increase in G-units would be expected to exacerbate lignin’s condensation, complexity and thus

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**Figure 7 | Enzymatic hydrolysis and bioconversion of \textit{MOMT4} transgenic woods to ethanol.** (a) Digestive weight loss when the prepared wood cell walls were treated with 2 mg ml\(^{-1}\) of cellulase for 72 h at 37 °C. Data represent mean ± s.e. with three biological replicates (each with two to three technical repeats) for the control and three technical repeats for transgenic lines. CWR, Cell wall residues. **P<0.01 (Student’s t-test). (b,c) Broth weight loss (Fermentation gas release) (b) and ethanol yield (c) during simultaneous saccharification and fermentation of the pretreated and untreated control and \textit{MOMT4} transgenic woods. Data represent mean ± s.d. of three biological replicates (each replicate with two experimental repeats for each control and transgenic lines. *P<0.05, **P<0.01 (Student’s t-test).
These results suggest that expression of comparison with the controls (Fig. 7; Supplementary Fig. 5). treatment, and up to 49% more ethanol resulted from the transgenic cell walls than from the control wood under the same ethanol. Up to 60% more woody biomass could be digested from in sugar release and had an enhanced bioconversion rate to transgenics’ woods displayed substantially improved efficiency recalcitrance of the cell walls. Surprisingly, the MOMT4 transgenics’ woods displayed substantially improved efficiency in sugar release and had an enhanced biorefinery rate to ethanol. Up to 60% more woody biomass could be digested from transgenic cell walls than from the control wood under the same treatment, and up to 49% more ethanol resulted from the conversion of the non-pre-treated transgenic woods, in comparison with the controls (Fig. 7; Supplementary Fig. 5). These results suggest that expression of MOMT4 in hybrid aspen leads to a significant alleviation of the recalcitrance to wood processing in an unexpected way, facilitating the conversion of woody biomass to liquid fuels. The improved wood digestibility of MOMT4 transgenic aspen may be attributed to several concurrent factors. For example, (1) the modest reduction of total lignin content in the cell wall that mitigates its physical barrier properties; (2) drastic structural alteration in lignin that may affect the association of lignin with polysaccharides and the interaction between cell wall biopolymers, thus changing the accessibility of the wall to digestive enzymes; (3) the relative increase of the content of cellulose fibres due to the cell wall’s mass balance, which, in turn, enhances the source of fermentable six carbon sugars. Interestingly, altering lignin content and structure not only promoted cellulose deposition but also altered its ultrastructure; more crystalline cellulose microfibrils were formed in the MOMT4 transgenics’ cell walls (Fig. 6). The underlying mechanisms for such concomitant alteration in polysaccharide deposition and structure remain unclear. One hypothesis is that S-lignin subunits and/or their linked hydroxybenzoate esters may play structural roles in the lignin–polysaccharide association. Disturbing S-lignin synthesis may trigger a potential feedback remodelling of cell wall carbohydrates. Alternatively, S-lignin deposition is regarded as one of the integral steps of plant secondary cell wall formation41; disrupting S-lignin synthesis may disturb or delay the cell wall’s development processes, sustaining wood at a younger stage, and thus rendering it less recalcitrant to breakdown. Collectively, our study suggests that lignin condensation does not necessarily correlate to the digestibility of Populus, and it is not the sole or critical factor dominating cell wall recalcitrance.

Expression of MOMT4 in the herbaceous dicot Arabidopsis, and the woody species, aspen, yields quite different metabolic and biological consequences. When MOMT4 expressed in Arabidopsis, the plants produced both the 4-O-methylated feruloyl and sinapoyl malates in their soluble phenolic fraction25, suggesting that the 4-O-methylated products of MOMT4 entered the endogenous pathway of phenolic-ester biosynthesis that is specific for Brassicaceae species42,43. The total lignin content in the stem of transgenic Arabidopsis showed a significant reduction, while its composition and structure essentially remained unchanged25. In contrast, when MOMT4 was expressed in aspen, the transgenic plant accumulated glucoconjugates of 4-O-methylated monolignols (Supplementary Fig. 1); furthermore, the lignin from transgenic tree was composed of a significantly lower level of S-subunits, and more condensed (C–C bonded) structures containing G-subunits (Figs 2 and 3) than occurred in the control aspen. This biological discrepancy may reflect the diversity and speciation of the phenylpropanoid metabolism, and the detoxification mechanism that the particular plants adopt.
and also the species variability of plant lignifications. In particular, in terms of the distinct effects of MOMT4 on lignin synthesis in these two species, *Populus* hardwood is rich in syringyl lignin with an S/G ratio ranging from 1.8 to 2.3\(^4\), whereas the lignin of *Arabidopsis* is guaiacyl rich (with S/G ratio \(\sim 0.25\))\(^5\). Since MOMT4 displays a discernible, yet less dominant, substrate preference to sinapyl alcohol, when it is expressed in the guaiacyl-rich *Arabidopsis*, its kinetic effect may be masked or compromised. Whereas when MOMT4 acts in an environment in which the S-monomeric substrate is more prominently available, its kinetic propensities may result in its dominantly modifying sinapyl alcohol, and thus triggering a metabolic consequence entailing a more severe disruption of S-lignin formation. Such metabolic preference in fact was also evidenced with the higher level of accumulation of the 4-O-methylated sinapyl alcohol than the 4-O-methylated coniferyl alcohol in the soluble phenolic fraction of transgenic aspen leaves and stems (Supplementary Table 1). Another possibility for preferentially reducing S-lignin in the transgenic aspens is that the MOMT4-produced 4-O-methylated compounds may exert inhibitory effects on the activities of *Populus* intrinsic S-lignin-specific enzymes. However, our *in vitro* study revealed that the 4-O-methylated monolignols have no detectable inhibitory effects on the activities of Cald5H/F5H or COMT from aspen; they only displayed a 3–14% inhibition on the activities of cinnamyl alcohol dehydrogenases (Supplementary Table 3). With such an inhibition, both the activities of CAD/SAD to the G- and S-lignin precursors should be affected, which do not explain the observed preferential impairment of S-lignin synthesis (Supplementary Table 3). The exact reasons for expressing MOMT4 in aspen preferentially to disrupt S-lignin formation may be complicated. It remains to be determined whether this phenomenon is *Populus* specific or occurs in the other woody species.

Despite the drastic change in lignin and cell wall structure, those greenhouse-grown, \(~6\)-month-old MOMT4 transgenic aspens showed no penalty in biomass yield, nor loss in wood density. The structural integrity at the whole plant- and cellular-level was maintained essentially similar to that of the control (Fig. 8). The sustained growth and fitness features of MOMT4 transgenic aspens are most likely attributed to their attaining condensed G-lignin, the increased cellulose content, and the elevated crystallinity of cellulose fibres in their cell walls along with a concomitant decrease in S-lignin formation. We note that the current study, examining the plant growth property, was conducted in a relatively short period. An extended physiological investigation, or a field trial with the generated transgenic trees, will substantiate their viability as biofuel feedstocks.

In summary, expressing an engineered monolignol 4-O-methyltransferase in hybrid aspen substantially alters lignin structure while also producing more cellulose. Such alteration facilitates wood digestibility and its bioconversion to ethanol, proving a useful strategy to tailor woody biomass for bio-based applications. The created transgenic aspen represents a valuable feedstock for further industrial exploitation in biofuel production.

**strain GV3101 for plant transformation. Hybrid aspen clone INRA 717-I74 ( *P. tremula × P. alba*) was propagated and transformed using the protocol of Ma et al.\(^7\). Briefly, leaf disc and stem segment explants, after wounding with multiple fine cuts, were co-cultivated for 1 h with *A. tumefaciens* strain GV3101 containing the MOMT4 expression construct. Then, the explants were cultivated on callus induction medium (CIM, 0.025% (w/v) 2-(N-morpholino)ethanesulfonic acid (MES), 0.01% (w/v) myo-inositol, 0.43% (w/v) ½ MS basal salts, 2% sucrose, 10 μM 2-i-naphthaleneacetic acid, 30 μM benzyladenine, pH 5.8) at 22 °C for 2 days. After washing with distilled water, the explants were subsequently cultured in dark on CIM containing 10 mg·l\(^{-1}\) hygromycin and 200 mg·l\(^{-1}\) timentin for selecting the transformed calli. After 2–3 weeks, the explants were sub-cultured on shoot selection medium (0.025% (w/v) MES, 0.01% (w/v) ½ MS basal salts, 3% sucrose, 1% (w/v) FV vitamins) containing 0.2 μM thidiazuron, 10 mg·l\(^{-1}\) hygromycin and 200 mg·l\(^{-1}\) timentin for 2–3 months and sub-cultured every 3–4 weeks. The explants with multiple shoots were then transferred onto the shoot selection medium supplemented with 0.1 μM 6-benzylaminopurine, 10 mg·l\(^{-1}\) hygromycin and 200 mg·l\(^{-1}\) timentin for shoot elongation. The regenerated shoots were transferred to the pots, and maintained in the greenhouse at 25 °C under high illumination at 500 μmol m\(^{-2}\) s\(^{-1}\). When the plants were fully established, they were then sub-cultured by natural light. The transgenic and control plants intended for lignin and phenolic analysis were grown side by side.

**Methods**

**Overexpression of MOMT4 in hybrid aspen.** Approximately 1.1 kb of the promoter of PAL2 from bean (*Phaseolus vulgaris* L.) was amplified from the vector pCAMBIA2200-GW (ref. 14) with a pair of primers that incorporated HindIII and KpnI restrictive enzyme sites, respectively (Supplementary Table 5). The 355 promoter of the vector pMDC32, a gateway destination vector\(^46\), was removed by the enzymatic digestion with HindIII and KpnI and replaced with bean PAL2 promoter; this yielded a binary vector pMDC32-PAL-GW. The open reading frame of the engineered MOMT4 gene was then integrated into the created binary vector following the standard gateway cloning procedures (Invitrogen). The resulting MOMT4 expression construct was transferred into *Agrobacterium tumefaciens*. The lignin of *Arabidopsis* is guaiacyl rich (with S/G ratio \(\sim 0.25\)). Since MOMT4 displays a discernible, yet less dominant, substrate preference to sinapyl alcohol, when it is expressed in the guaiacyl-rich *Arabidopsis*, its kinetic effect may be masked or compromised. Whereas when MOMT4 acts in an environment in which the S-monomeric substrate is more prominently available, its kinetic propensities may result in its dominantly modifying sinapyl alcohol, and thus triggering a metabolic consequence entailing a more severe disruption of S-lignin formation. Such metabolic preference in fact was also evidenced with the higher level of accumulation of the 4-O-methylated sinapyl alcohol than the 4-O-methylated coniferyl alcohol in the soluble phenolic fraction of transgenic aspen leaves and stems (Supplementary Table 1). Another possibility for preferentially reducing S-lignin in the transgenic aspens is that the MOMT4-produced 4-O-methylated compounds may exert inhibitory effects on the activities of *Populus* intrinsic S-lignin-specific enzymes. However, our *in vitro* study revealed that the 4-O-methylated monolignols have no detectable inhibitory effects on the activities of Cald5H/F5H or COMT from aspen; they only displayed a 3–14% inhibition on the activities of cinnamyl alcohol dehydrogenases (Supplementary Table 3). With such an inhibition, both the activities of CAD/SAD to the G- and S-lignin precursors should be affected, which do not explain the observed preferential impairment of S-lignin synthesis (Supplementary Table 3). The exact reasons for expressing MOMT4 in aspen preferentially to disrupt S-lignin formation may be complicated. It remains to be determined whether this phenomenon is *Populus* specific or occurs in the other woody species.

**Methanolic soluble phenolic profiling.** Approximately 2-month-old plantlets of the control lines and the MOMT4 transgenic lines were harvested. All the leaves on a shoot of about 30 cm (above ground), and the shoot itself (without bark) were collected from individual lines and ground into fine powders under liquid nitrogen. Approximately 0.2 g of the leaf or stem powders were extracted overnight with 1 ml 80% methanol (containing 20 μM chrysin as the internal standard). Then, 25 μl of the obtained extract or 200 μl extracts after solvent evaporation were injected with 2 μl·m\(^{-1}\) 1-β-gulosiode in 50 μl citric-phosphate buffer (pH 7.0), and then re-extracted with ethyl acetate. After drying them under liquid nitrogen for 10 min, the ethyl acetate extracts were re-dissolved in 100 μl methanol, and 30 μl was taken for HPLC analysis. For ultraviolet-HPLC profiling, the samples were resolved in a mobile phase of 0.2% acetic acid (A) with an increasing concentration gradient of acetonitrile containing 0.2% acetic acid (B) at 0 to 2 min, 5% (B); 2–30 min, 5 to 50% (B); 30–32 min, 50 to 100%, and then 100% for 2 min at a flow rate of 1 ml·min\(^{-1}\). The ultraviolet absorption was monitored at 254-, 280-, 310- and 330-nm using a multiple-wavelength photodiode array detector. The detected compounds were identified based on the peak retention time and calculated against the standard curves of coniferyl alcohol and sinapyl alcohol as they exhibit nearly the same ultraviolet absorption property as do their 4-O-methylated compounds.

The 4-O-methylated coniferyl alcohol and sinapyl alcohol used for identifying the leaf metabolites were synthesized by the MOMT4-catalysed reactions as described below in the section of Enzyme activity inhibition assay.

For characterizing the novel metabolites by GC–MS, the leaf extracts after β-glucosidase digestion were re-extracted with ethyl acetate. After drying under N\(_2\) gas, the residuals from the extracts were derivatized with 30 μl pyridine and 30 μl MSTFA at room temperature for 4 h, and then dried under a stream of N\(_2\) gas. Dichloromethane (60%) were used to dissolve the samples. Around 1 μl was injected for GC–MS analysis. The mass spectra of two distinct metabolites in the chromatograms of transgenic samples were recorded.

**Histological analyses.** Histological analyses was performed on 30-μm-thick sections taken from the same position of basal node of 2-month-old shoots of the control and MOMT4 transgenic aspens. For phloroglucinol (Wiesner) staining, the fresh sections were left for 5 min in 1% phloroglucinol in 95% ethanol and mounted in 6 N HCl. For Ma–LeucTT staining, the stem sections were immersed in 1% (w/v) potassium permanganate solution for 5 min at room temperature and then washed with water and acidified with 3% hydrochloric acid until partially decoloured. Sections were finally mounted in 50% NaHCO\(_3\) and examined quickly. Photographs were taken by using a Leica stereomicroscope system with a Leica DFC 300 colour camera.

**Analysis of wall-bound phenolics and lignin.** Aspen stems were harvested from 6-month-old plants. Segments of basal stems \(\sim 30\) cm long, taken from each of the transgenic and control lines, were peeled to remove bark, dried and then ground in a Wiley mill. The milled wood chips were passed through a 60-μm mesh sieve. The
wood powders were then extracted with 70% ethanol at 65 °C for 3 h. This process was repeated for three times. The residuals were further extracted three times with chloroform/methanol (CFM) (1:1, v/v), and then dried under vacuum at room temperature. The extracted free residuals were dried at 45 °C and then ball-milled into a fine powder. The wall-bound phenolics were extracted with 2 N NaOH in the dark at 37 °C for 16 h. After neutralization of the reaction with concentrated HCl, the hydrolysate was extracted with water-saturated ethyl acetate twice. The extract was dried under stream of N2 gas then dissolved in 100 μl 80% methanol and analysed using HPLC with the aforementioned method (in soluble phenolics profiling section).

Total lignin was quantified by the acetyl bromide method46. Extractive-free wood powders (5 mg) were incubated with 1 ml 75% acetyl bromide in acetic acid at 70 °C for 30 min. After cooled down and diluted with 5 ml acetic acid, 300 μl solution was aliquoted and neutralized with 400 μl 1.5 M NaOH and 300 μl 0.5 M hydroxylamine hydrochloride. The neutralized sample was further diluted with 1.5 ml acetic acid and the absorbance at 280 nm was measured. The extinction coefficient of 18.21 g−1 cm−1 was used for the calculation of lignin content.

The thiocyanidolysis method46 was followed to estimate the monomeric composition of lignin. Briefly, for each sample, 10 mg of extractive-free wood powders were mixed with 1 ml of freshly prepared reaction mixture (2.5% boron trifluoride etherate and 10% ethanethyl in distilled dioxane (v/v)) in a 2 ml glass vial and sealed with N2 gas. Then the vial was tightly sealed and heated at 95 °C for 4 h with periodic agitation. The reaction was stopped by placing on ice for 15 min and then its pH value was brought to 3–4 by using 0.4 M sodium bicarbonate. The reaction solution was transferred to a new 10 ml vial; 2 ml water was added. Meanwhile, 1 mg tetrascosate (dissolved in 1 ml methylene chloride as internal standard) was added to each vials. The vial was recapped, vortexed, then allowed to stand without stirring for 10 h and half hour before the separation of phases occurred. An aliquot (1.5 ml) of the organic phase was taken and passed through a Pasteur pipette packed with an inch of anhydrous sodium sulfate. The filtrate was then evaporated to the dryness and resuspended in 0.5 ml of methylene chloride. Samples (50 μl) were then dried and derivatized with pyridine and N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma) at room temperature for 5 h. Quantifying the corresponding monomers was accomplished via a gas chromatography–flame ionization detector on a GC instrument (Agilent 7890A) after an appropriate calibration relative to the tetrascosate internal standard.

**Whole-cell wall NMR analyses.** The pre-ground cell walls were extracted with distilled water (ultrasonication, 1 h, three times) and 80% ethanol (ultrasonication, 1 h, three times). Isolated cell walls were dried and ball-milled in a Planetary micro mill Pulverisette 7 premium line (Fritsch, Idar-Oberstein, Germany) at 600 rpm, using ZrO2 vessels (20 ml) containing ZrO2 ball bearings (10 mm) and gels with N2 gas. The vial was recapped, vortexed, then allowed to stand without stirring for 10 h and half hour before the separation of phases occurred. An aliquot (1.5 ml) of the organic phase was taken and passed through a Pasteur pipette packed with an inch of anhydrous sodium sulfate. The filtrate was then evaporated to the dryness and resuspended in 0.5 ml of methylene chloride. Samples (50 μl) were then dried and derivatized with pyridine and N-methyl-N-(trimethylsilyl)trifluoroacetamide (Sigma) at room temperature for 5 h. Quantifying the corresponding monomers was accomplished via a gas chromatography–flame ionization detector on a GC instrument (Agilent 7890A) after an appropriate calibration relative to the tetrascosate internal standard.

**FT-IR spectroscopic imaging**. Infrared images of the sections of poplar stems followed the method described by Gou et al.55. Briefly, a piece of aspen stem from ~1-month-old plantlet within the first five internodes was cut and fresh-frozen over liquid nitrogen. Then, the specimen was embedded in Tissue-Tek freezing medium and sectioned with a cryomicrotome (Leica Microsystems) at ~20 °C. Sections (10 μm thick) were placed on a BaF2 microscope slide and dried at room temperature. IR images were collected using a Perkin Elmer Spectrum Spotlight FT-IR Imaging System (Waltham, MA, USA) with a 6.25-μm pixel resolution. For each tissue section, a light micrograph was obtained, and the regions to be imaged with the IR microscope were defined. Infrared images were collected in the transmission mode by raster-scanning the sample through the IR beam, and collecting infrared absorbance spectra at each pixel. For each pixel, an entire infrared spectrum is obtained from 4,000- to 720 cm−1. Background spectra were gathered from a clean region of the BaF2 slide. The spectral resolution was 8 cm−1, and 16 scans were averaged for each spectrum.

The corresponding FT-IR image was created by visualizing the intensity of the spectra at 1,130 cm−1, which was assigned to the vibration of the phenolic ring from lignins56 and the spectra bands from 900- to 1,180-cm−1, representing the overlapped polysaccharides. Both sets of images were normalized to the C–H intensity to correct for any variation in sample thickness; empty pixels covering cells’ lumens were eliminated by setting a threshold limit for the absorbance at lignin and polysaccharides.

**Determination of lignin molecular weight.** The lignin fractions were prepared essentially as described57 with minor modifications. Briefly, the prepared poplar cell walls were further intensively milled using a Retch ball mill PM100 for 5 × 10 min with 5 min pause in between. The ball-milled materials were extracted twice with 96×4 dioxane-water. The extracts were pooled and freeze-dried, resulting in the milled wood lignin (MWL). The insoluble materials were then treated at 30 °C with a crude cellulase mixture (Onozuka R-10, PhytoTechnology Laboratories). The materials were extracted twice more with 96×4 dioxane-water. The extracts were pooled and freeze-dried, giving rise to so-called cellulolytic enzyme lignins (CEL). The remaining insoluble materials were referred to as residue lignin (RL). The different lignin materials were then dissolved in enzyme lignins (CEL). The remaining insoluble materials were referred to as residue lignin (RL). The different lignin materials were then dissolved in

**Cellulose quantification and cellulolytic analysis**. Samples (50 μl) were acetylated with acetic anhydride (0.6 %, methylimidazole (NMI) and dimethyl sulfoxide in a ratio of 1:2 and acetylated by heating at 70 °C for 16 h. After neutralization of the reaction with concentrated HCl, the alditols were incubated with acetic anhydride and 1-methylimidazole for acetylation. The resultant alditol acetates were finally dissolved in dichloromethane and separated on a gas chromatograph that was equipped with a 30 m × 0.25 mm (internal diameter) Agilent J&W HP-5MS capillary column, and the eluant introduced into a mass spectrometer (Agilent Technologies); the initial oven temperature (250 °C) was maintained at 250 °C for 30 min, increased to 320 °C at 5 °C min−1 and then increased to 230 °C at 2 °C min−1 and held there for 5 min. The individual sugars were identified by comparison with authentic standard compounds; their quantification was based on the standard curves of derivatized individual sugar made from the same GC–MS run.

**Fermentation of aspen wood.** Basal stems (~30 cm) from 6-month-old newly propagated MOMT4 transgenic and control plantlets, or from ~1-year-old primary transgenic trees after three coppice events were harvested, air dried and milled to fine powder (passing through a 60-μm mesh sieve). The materials were used directly for fermentation or were pretreated as follows: the wood powders were mixed with 1% Ca(OH)2 at the ratio of 10% (w/v) biomass, and treated at 121 °C for 90 min. After cooling, the treated powders were collected by centrifuging, and then washed three times using distilled water in 2 ml dH2O/g wood biomass to remove the soluble materials. The pretreated wood powders then were completely dried at
45°C. Simultaneous saccharification and fermentation was undertaken following an established protocol36 with slight modification. Briefly, the fermentation was carried out using CDAP capillary and autoclaved bottles containing 1% (w/v) wood biomass (either pretreated or untreated), 1% (w/v) yeast extract, and 2% (w/v) bactopeptone in 50 mM sodium citrate buffer (pH 4.8). After autoclaving them, we added asealosterone 1500 (DuPont, WI, USA) that contains multiple enzyme activities, including endoglucanase (400 U/mL CM C 1.5), and β-glucosidase (450–775 nPNU U g1−1), at 0.3 mL g−1 biomass, and yeast (Saccharomyces cerevisiae D5A, ATCC 200062) at a final OD600 of 0.5 in the total fermentation reaction volume of 12.5 mL. The bottle was incubated at 37°C in a shaker at 175 r.p.m. to prevent sedimentation of the substrate, and the yeast. At the indicated incubation time, the sample was diluted (10 fold); the inoculum was diluted with a split ratio of 1:10, the n-Propanol was used as an internal standard for calibration. We used the standard curve of ethanal made in the same GC-FID run for quantifying the yield of fermentation ethanal.

Wood density. Approximately 8-month-old control plants and MOMT4 primary transgenic plants were harvested. After removing their bark, the primary stems were then dried at 45°C. The basal part of the stem of each line was then cut into three sections (each ~5 cm), the dry weight (Ws) of each section was measured at zero; the volume of each section was then measured in a graduated cylinder filled with fine glass beads (soda lime with diameter of 0.5 mm, Biconspect Products). The volume of glass beads before and after inserting stem section was recorded and termed V0 and V1, respectively. The volume of each sample was calculated as V = V1 – V0. The density of the wood was calculated as D = Ws/(V1 – V0). The data from six control lines and eleven transgenic lines were averaged, respectively.

Enzyme activity inhibition assay. To prepare 4-O-methylated monolignols, the purified MOMT4 enzyme was incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 2 mM coniferyl alcohol or sinapyl alcohol, 10 mM SAM and purified MOMT4 enzyme was incubated in a reaction mixture containing 50 mM sodium phosphate (pH 7.2) containing 20% glycerol and 4 mM disodium EDTA) and stored at ~8°C. PrtCOMT was cloned from Populus trichocarpa and its recombinant enzyme was expressed and produced in Escherichia coli BL21(DE3) strain.

In vitro polymerization assay. 500 μL coniferyl alcohol or sinapyl alcohol was incubated with 40 ng horseradish peroxidase (Sigma), and 1.14 mM H2O2 in 25 mM Tris-Ci buffer (pH 7.5) in the presence or absence of 40 μM 4OxMeCA or 4-O-methylated -coniferyl alcohol or -sinapyl alcohol to examine their potential inhibition on the activity. The enzymatic products were profiled using HPLC following the method described above.

qRT-PCR analyses of monolignol biosynthetic genes. The 4th–13th internodes of 2-month-old developing stems were harvested from two independent control lines and two MOMT4 transgenic lines (MOMT4-0 and MOMT4-2). For each line, two individual plants were respectively sampled. The total RNAs were extracted using a CTAB-based method36. First strand cDNA was synthesized using M-MuLV Reverse Transcripase (New England Biolabs) under the manufacture recommended conditions. The selection of monolignol biosynthetic genes and primer designs were performed as described35 except PrtFT1 (ref. 64) was used as the reference gene. For each sample, qRT-PCR were carried out in triplicates for all the genes examined with primers listed in Supplementary Table 5. SsoAdvanced DNA polymerase (Biorad) was used for the reaction. The cycle parameters were performed as described35 except the reference gene. For each sample, qRT-PCR were carried out in triplicates for all the genes examined with primers listed in Supplementary Table 5. SsoAdvanced DNA polymerase (Biorad) was used for the reaction. The cycle parameters were performed as described35 except the reference gene.

RNA sequencing. The 1st to 15th internodes from the top of 2-month-old hybrid aspen stems of MOMT4-0 transgenic and control lines were harvested and, after removing their bark, they were immediately frozen in liquid nitrogen. About 100 mg stem samples pooled from 2 to 3 individual transgenic and control plants, respectively, were grounded to fine powder under liquid nitrogen. Total RNAs were extracted using Qiagen RNeasy Plant Mini Kit (#Cat. 74903) following the manufacturer recommended conditions. The selection of monolignol biosynthetic genes and primer designs were performed as described35, except PrtFT1 (ref. 64) was used as the reference gene. For each sample, qRT-PCR were carried out in triplicates for all the genes examined with primers listed in Supplementary Table 5. SsoAdvanced DNA polymerase (Biorad) was used for the reaction. The cycle conditions were performed as described35 except the reference gene. Following these alignments, raw counts for each poplar gene were derived and normalized to reads per kilobase of exon model per million mapped reads (RPKM). The differentially expressed genes were identified with the integrated Cuffdiff programme41,42 based on False Discovery Rate adjusted p value (that is, q value) at the cutoff of 0.05.

Statistical analysis. Statistical analysis was performed by Student’s t-tests (two-tailed distribution and two-samples with unequal variances). Statistical values were expressed as means with s.d. or s.e. Microsoft Excel 2011 (Microsoft Corporation) were used for data management, statistical analysis, and graph generation.
Data availability. RNA-seq data described in this study has been deposited in the GEO Database under accession code GSE11213. The authors declare that all other data supporting the finding of this study are available within the article and its Supplementary Information files or are available from the corresponding author upon request.

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Author contributions
C.-J.L. designed and oversaw all of the studies, analysed and interpreted data, and wrote the manuscript. K.Z. designed and conducted aspen transformation, transgenic plant analyses, and cellulolytic measurement. Y.C. conducted cellulose content measurement, fermentation study, and enzyme activity inhibition assays. H.Y. maintained transgenic plants and conducted RNA-seq analysis. X.Z. characterized soluble phenolics. J.R. and H.K. performed NMR analyses and data interpretation. G.H. conducted scan electron microscopy study. M.L. assisted in synchrotron FT-IR imaging. All authors edited the manuscript.

Additional information
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