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Improved chemical pulping and saccharification of a natural mulberry mutant deficient in cinnamyl alcohol dehydrogenase

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Title: Improved chemical pulping and saccharification of a natural mulberry mutant deficient in cinnamyl alcohol dehydrogenase

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Abstract

Lignin content and its molecular structure influence various wood characteristics. In this study, the anatomical and physicochemical properties of wood derived from a naturally occurring mulberry mutant deficient in cinnamyl alcohol dehydrogenase (CAD), a key enzyme in lignin biosynthesis, were analyzed using conventional staining assays on stem sections, length and width measurements of xylem fiber cells, wood pulping and saccharification assays, and sugar compositional analysis of extractive-free wood powder. The present data indicate that the mutation in the *CAD* gene leads to improved wood delignification efficiency, increased pulp yield under alkaline pulping conditions, and enhanced saccharification efficiency following alkaline pretreatment. This study opens up new avenues for the multipurpose use of the mulberry CAD-deficient mutant as a raw material for biorefinery processes, in addition to its traditional use as a favored feed for silkworms.

Keywords: alkaline pulping, cell wall, enzyme saccharification, lignin, lignocellulose

1. Introduction

Mulberry trees are widely cultivated for their fruit, for silk production, and as fodder for livestock (Ercisli et al. 2007; Hamamura 1959; Saddul et al. 2004). Mulberry wood also has great potential as a feedstock for biofuel and chemical pulp production in rural areas, especially in places where sericulture is well-developed (Guha et al. 2013; Lu et al. 2009; Rahman and Jahan 2014; Cao et al. 2019). Notably, mulberry leaves are an excellent feed for silkworms (*Bombyx mori*), as they contain all the essential proteins, carbohydrates, and vitamins for silkworm growth and development. After the emergence of synthetic fibers in industrialized countries, sericulture became less important, although it is still a major agricultural industry in some countries, such as China and India.

According to Yoshimura and Saito (1924), a farmer discovered a mulberry tree with drooping branches and unusual red-colored wood in bushland on Okushiri Island, Hokkaido, northern Japan, around the year 1912. The tree was named Sekizaisou, which means “mulberry with red-colored wood” in Japanese, by Yoshimura and Saito (1924). Grafted Sekizaisou plantlets were established a century ago and have been preserved ever since by sequential vegetative propagation.

Based on chemical and genomic experiments, Sekizaisou has been identified recently as a natural mutant deficient in cinnamyl alcohol dehydrogenase (CAD), which catalyzes the reduction of hydroxycinnamaldehydes to their hydroxycinnamyl alcohols (monolignols), the last step in the monolignol biosynthetic pathway (Yamamoto et al.

2020). A guanine insertion close to the predicted translation start codon of the *CAD1* gene in Sekizaisou caused a frameshift and, consequently, a premature stop codon in the putative first exon of the gene, suggesting production of a nonsense peptide with only 29 amino acid residues. The red xylem coloration, as observed in Sekizaisou, is a typical characteristic of mutants and transgenic plants with altered lignin structure (Baucher et al. 1996; Kajita et al. 1996; Leplé et al. 2007; Ralph et al. 1997; Sattler et al. 2010; Sibout et al. 2005; Van Doorsselaere et al. 1995; Voelker et al. 2010; Zhang et al. 2006). Without any chemical staining, the fresh xylem tissues in such mutant and transgenic plants are brown-, orange-, or red-colored. Among the many mutants and transgenic plants showing modified lignin structures, only two natural woody (non-transgenic) mutants with homozygous mutations in lignin biosynthetic genes have been described, namely loblolly pine (MacKay et al. 1997) and poplar (Vanholme et al. 2013), displaying premature stop codons in the *CAD* gene and a hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (*HCT*) gene, respectively. Sekizaisou is the third instance of natural woody mutant with a defect in a gene involved in monolignol biosynthesis and the first reported homozygous dicotyledonous tree deficient in CAD.

Lignin plays crucial roles in the conduction of water through vascular tissues and in providing strength and rigidity to support the plant body (Boerjan et al. 2003). Despite its importance to plant growth and development, lignin is a major barrier to the biorefining of lignocellulosic materials. It is naturally made by the polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols, the so-called monolignols, via their phenolic radicals, producing *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in polymeric lignin (Boerjan et al. 2003; Freudenberg and Neish 1968; Ralph et al. 2019; Vanholme et al. 2019). The incorporation of alternative monomers into lignin, caused by either natural mutations, perturbation of the lignin biosynthetic pathway, or by metabolic engineering of exotic phenylpropanoids, can reduce lignin recalcitrance and may change the color of xylem tissue (Mottiar et al. 2016; Oyarce et al. 2019; Sakamoto et al. 2020; Vanholme et al. 2012; Wilkerson et al. 2014).

Sekizaisou, as well as transgenic poplar and tobacco plants with reduced *CAD* expression, and *Arabidopsis* and pine mutants deficient in CAD, have a slight decrease in lignin amount and also exhibit the colored xylem phenotype (Halpin et al. 1994; MacKay et al. 1997; Pilate et al. 2002; Ralph et al. 1997; Sibout et al. 2005; Van Acker et al. 2017; Yamamoto et al. 2020). Phenylpropanoids from the canonical monolignol pathway, and pathways branching therefrom, significantly accumulated in Sekizaisou. Heteronuclear single-quantum correlation spectroscopy ($^{1}H–^{13}C$ HSQC) revealed that coniferaldehyde and sinapaldehyde were integrally coupled into the lignin polymer in

Sekizaisou at much higher levels (Yamamoto et al. 2020). CAD-deficient transgenic plants and pine *cad-null* mutants exhibit higher delignification efficiency in chemical pulping as compared to control plants (Chanoca et al. 2019; Dimmel et al. 2001; Lapierre et al. 1999; O'Connell et al. 2002; Pilate et al. 2002). Here, in addition to anatomical characteristics, the pulping and saccharification efficiencies of Sekizaisou wood in comparison with other mulberry cultivars have been examined. The present data reveal that Sekizaisou wood is a promising raw material for biorefineries.

2. Materials and methods

2.1 Preparation of wood chips

Branching stems (1-year-old) of 5 mulberry cultivars (Sekizaisou, Chosenzairaishu, Kataneo, Kinsou, and Nezumigaeshi) were harvested in winter of 2017 at the experimental field of the National Agriculture and Food Research Organization, Tsukuba, Japan (36°03N 140°05E). All cultivars are classified as *Morus alba* (Machii et al. 2001). Bark from the stems was peeled off using a hand knife, and then the stems were cut into short sticks, as shown in Figure 1. After being air-dried at room temperature, the wood sticks were further cut into small pieces (approximately 1 cm in length) using secateurs and then chopped in half vertically using a hand knife to prepare wood chips for use in chemical pulping trials.

2.2 Histochemical analysis with Wiesner and Mäule staining

Fresh stem samples were fixed in a formaldehyde/acetic acid/alcohol (FAA) solution (50% ethanol, 10% formaldehyde, and 5% acetic acid). Small stem segments were dehydrated in a graded ethanol series (50%, 60%, 80%, 90%, and 100% v/v), and were then embedded in LR White Hard (TAAB, Aldermaston, UK) with 5% PEG400. Cross sections of 3 µm thickness were cut using a Leica Jung RM2055 microtome (Leica Microsystems, Wetzlar, Germany). Sections were stained with a 0.5% toluidine blue solution and mounted in 'Entellan® new', a rapid non-aqueous mounting medium (Merck KGaA, Darmstadt, Germany). Cross sections of 20 µm thickness were subjected to lignin staining. For Wiesner staining (Adler et al. 1948), sections were stained with 0.1% phloroglucinol in an ethanol/HCl solution (25 mL of ethanol and 4 mL of concentrated HCl). For Mäule staining (Meshitsuka and Nakano 1977; Nuoendagula et al. 2018), sections were stained with 1% $KMnO_4$, and then washed with distilled water three times. After incubation in 3% HCl, sections were washed with distilled water and colored in 29% NH_4OH. Section images were captured by a Leica DMR microscope with a Leica

MC170HD microscope camera (Leica Microsystems).

2.3 Wood density

Wood density was calculated according to procedures described by Nuoendagula et al. (2018). The bark and pith were removed from the stem samples fixed in FAA. Three segments were obtained from each xylem sample, and then the fixative was replaced with ultrapure water at ambient temperature for three days. Segment volume was measured by Archimedes' principle at 20 °C using a precision balance (Secura124-1S; Sartorius, Göttingen, Germany). The samples were dried in a heating oven at 105 °C for three days, and then the dry weights of the samples were measured using a precision balance. Wood density was calculated by dividing dry weight by volume.

2.4 Fiber length and width

Xylem samples without bark or pith were macerated in a solution of 6% hydrogen peroxide and glacial acetic acid (1:1) at 95 °C for 8 h (Franklin 1945). After being washed with ultrapure water, the samples were neutralized by sodium carbonate. The samples were washed with ultrapure water, and then defibrated by vigorous shaking. Wood fibers were visualized using a Leica DM6000B microscope with a Leica DFC310 FX microscope camera (Leica Microsystems). The lengths and widths of fiber cells were measured using the ImageJ software (n = 100).

2.5 Monosaccharide composition of mulberry wood

Monosaccharide composition was analyzed as described previously (Sakamoto et al. 2015). After cell wall residue was prepared by sequential extraction of pulverized wood with hot water and organic solvents, it was hydrolyzed by 4% sulfuric acid at 121 °C for 1 h. The supernatant was then neutralized by a calcium carbonate solution and labeled with ethyl *p*-aminobenzoate. Chromatographic separation and detection of the labeled monosaccharides were carried out using an ACQUITY UPLC H-Class liquid chromatography system equipped with an ACQUITY UPLC BEH C18 column (100 mm length × 2.0 mm inner diameter, 1.7 μm particle size; Waters, Inc., Milford, MA, USA) and a fluorescence detector (ACQUITY UPLC FLR Detector; Waters Inc.). Acetonitrile containing 200 mM potassium borate buffer (pH 8.9) was used as the eluent buffer.

2.6 Chemical pulping

After preparation of wood chips as described above, the cooking of the chips was performed as previously reported (Ikeda et al. 2007) in a small, stainless steel autoclave

(volume 150 mL) equipped with an oil bath. A mixture of air-dried wood chips (5 g of oven-dried weight equivalent) and sodium hydroxide solution (25 g) was placed in the autoclave, and then heated at 160 °C for 3 h (including preheating time). Two different concentrations of sodium hydroxide solution were applied during cooling: 25% (23.75 g of H_2O plus 1.25 g of NaOH) and 30% (23.5 g of H_2O plus 1.5 g of NaOH). The percentages of sodium hydroxide in the solutions are expressed based on wood chip weight (oven-dried weight equivalent), not on total volume of the solution. After cooking, the wood chips were defibrated using a homogenizer, and the resultant pulp fibers were separated from non-defibrated knots (incompletely cooked wood). The lignin contents of the pulp fibers were determined by a modified Klason method described previously (Yoshihara et al. 1984).

2.7 Alkaline pretreatment and subsequent saccharification

Saccharification efficiency was monitored after diluted alkali pretreatment. Extractive-free powder was prepared from woods of different mulberry cultivars by pulverization and subsequent solvent extractions. The powder (~10 mg) was mixed with 400 µL of 6.25 or 62.5 mM NaOH solution and heated at 120 °C for 1 h. After cooling to room temperature, the solution was neutralized with diluted hydrochloric acid. Saccharification was then carried out in 5 mM citrate buffer (pH 4.8) with Celluclast (Sigma-Aldrich C2730), Cellobiase (Sigma-Aldrich C6105), and 0.02% sodium azide at 50 °C for 24 h. Sugars released from the samples were quantified by DNS assay using glucose as a standard (Miller 1959). Sugar release (%, w/w) was calculated based on weights of released sugar and the extractive-free powder (air-dried for a month). Saccharification efficiency was also measured with samples without alkaline pretreatment and subsequent neutralization. The samples from three independent biological replicates were analyzed for each cultivar.

3. Results and discussion

3.1 Lignin characterization by chemical staining of thin sections

Sekizaisou was discovered as a natural bush; thus, there is no information on its parentage or genetic background. Therefore, a Nezumigaeshi cultivar whose morphological characteristics, such as leaf and fruit shape, bark color, and lenticel density, resembled those of Sekizaisou was used as a control in histochemical analyses (Machii et al. 2001). The xylem of Sekizaisou stems is bright red in summer (Yamamoto et al. 2020) and turns to faint pink in winter, when we prepared the samples for this study (Figure 1a).

Sekizaisou wood has a lower lignin content (14.9%, w/w) than that of other mulberry cultivars including Nezumigaeshi (22.3%) (Yamamoto et al. 2020). The syringyl to guaiacyl (S/G) ratio in the lignin of Sekizaisou (0.78) is also lower than those in other cultivars (1.01 in Nezumigaeshi) (Yamamoto et al. 2020).

The lignin was qualitatively analyzed using two different staining procedures, Wiesner and Mäule staining. Upon Wiesner staining, the cinnamaldehyde end-groups of lignin stain the xylem red-pink. Sekizaisou sections exhibited a stronger red color (Figure 1c) than did control sections (Figure 1g). Upon Mäule staining, which colors G units tan and S units red, a cross-section of a branching stem prepared from the control cultivar (Nezumigaeshi) presented a reddish-brown color (Figure 1h), whereas that from Sekizaisou exhibited a lighter color (Figure 1d). These results indicate structural alterations in Sekizaisou, in agreement with the 33% lower lignin content and the 16.5% increased frequency of sinapaldehyde incorporation in Sekizaisou versus Nezumigaeshi as reported by Yamamoto et al. (2020).

3.2 Fiber morphology and wood density

Based on images of toluidine blue-stained thin sections (Figures 1b and 1f), the size of xylem fibers in Sekizaisou seemed to be smaller than those in Nezumigaeshi. Fiber morphology and wood density have often an influence on biorefinery characteristics of wood and physical properties of pulp prepared from wood. To verify the potential differences, the length and width of defibrated fibers prepared by mercerization were measured. The length of Sekizaisou fibers was not significantly different from those of Kinsou fibers (Figure 2). In contrast, fiber lengths of the other 3 cultivars were significantly shorter than those of Sekizaisou and Kinsou. The fiber lengths determined in the present study are slightly shorter than those of mulberry wood pulp reported by Rahman and Jahan (2014). It might be due in part to the differences of cultivars used and/or of plant cultivation conditions in the different studies. Differences in fiber width were also observed among the cultivars tested. The width of Sekizaisou fibers was slightly but significantly larger than that of Kataneo. In contrast, the Sekizaisou fiber width was significantly smaller than those of other cultivars including Nezumigaeshi. This result is consistent with the observation of the cross sections analyzed by histochemical staining (Figures 1b and 1f).

In addition to the characterization of fiber morphology, the density of wood samples prepared from the stems of 5 different cultivars, including Sekizaisou, was measured using Archimedes' principle (Figure 3). The density of Sekizaisou samples was comparable with that of the other cultivars except for Nezumigaeshi samples that has a

significantly lower density. Rahman and Jahan (2014) reported that the densities of woods prepared from 8- and 12-month-old mulberry plants were 390 kg/m^3 and 410 kg/m^3, respectively. These values are similar to that of Sekizaisou (410 kg/m^3) and those of other cultivars reported in the present study. Collectively, the anatomical characteristics suggest that Sekizaisou wood has great potential as a raw material for chemical pulp, though the length of the fiber is relatively shorter than those of typical hardwood species such as *Eucalyptus* and *Acacia* commonly used in commercial pulp production.

3.3 Monosaccharide composition of xylem cell walls

Before the evaluation of pulping and saccharification performance, the monosaccharide composition of wood from each mulberry cultivar was compared. The results indicated that the monosaccharide compositions of all cultivars, including Sekizaisou, were typical of angiosperm woody plants, in which glucose and xylose are the major monosaccharides composing the polysaccharides of the cell wall (Table 1). The relative levels of glucose and xylose among the total detected monosaccharides released from the mulberry wood samples were comparable to those of other hardwood species.

Sekizaisou wood consisted of similar amounts of monosaccharides to the other cultivars analyzed (Table 1). Although differences in monosaccharides were observed among cultivars, they were not specifically attributed to Sekizaisou. The total amount of monosaccharides released from Sekizaisou wood was significantly lower than that from Kataneo, Chosenzairaishu, and Kinsou. The difference in the total amount was mainly due to variations in the amounts of glucose and xylose among the cultivars. In Arabidopsis, no significant difference in monosaccharide composition could be detected in *cad-6* (*cad-d*) mutants, whereas a small increase in the proportion of uronic acids (galacturonic and glucuronic acids) has been reported in the *cad-c/cad-d* double mutant (Thévenin et al. 2011; Van Acker et al. 2013). In a *CAD1*-deficient *Medicago truncatula* mutant, immunological glycome profiling revealed changes in the cross-linking of several classes of cell wall polysaccharides (Zhao et al. 2013), whereas increases in cellulose and pectin were induced by *CAD* downregulation via RNA interference in transgenic flax (Preisner et al. 2014).

3.4 Higher delignification efficiency of Sekizaisou wood

Reducing *CAD* expression in various transgenic plants improves lignin extractability and promotes subsequent polysaccharide recovery from lignified cell walls under different chemical treatments (Baucher et al. 1996; Bouvier d'Yvoire et al. 2013; Fu et al. 2011; Jackson et al. 2008; Lapierre et al. 1999; Martin et al. 2019; O'Connell et al. 2002; Pilate

et al. 2002; Segmehl et al. 2019; Straub et al. 2019; Van Acker et al. 2017; Wang et al. 2018). In addition, a nonsense mutation in the *CAD* gene of loblolly pine improved pulping performance of the mutant wood under alkaline pulping (Dimmel et al. 2001). To evaluate the delignification efficiency of Sekizaisou wood, we compared the chemical pulping performance of Sekizaisou wood chips with that of other cultivars using a soda pulping protocol under two different alkaline conditions (Table 2). Soda pulping is receiving increasing attention as an environmentally friendly biorefinery pretreatment process that limits sulfur emissions compared to the more recently commercialized kraft cooking process.

When pulping with a 30% NaOH solution at 160 °C for 3 h, a significantly higher pulp yield was achieved with wood chips from Sekizaisou and Kataneo than with wood chips from other cultivars (Table 2). The residual lignin content in the resultant pulp was significantly lower in Sekizaisou (5.3%, w/w) than in the other cultivars, except for Kinsou (6.2%). Fewer cooking knots (NC, uncooked dark aggregates of wood fibers) were detected in Sekizaisou pulp than in pulp of the other cultivars, although the difference was only statistically different between Sekizaisou and Kinsou ($P < 0.05$). With the less severe alkaline concentration (25% NaOH) under the same time and temperature conditions, Sekizaisou still produced significantly more pulp and less residual lignin than did the other cultivars, despite the lower total amount of detected monosaccharides in Sekizaisou compared to Kataneo, Kinsou, and Chosenzairaishu (Table 1). Strikingly, no knots could be found after cooking Sekizaisou wood under the less severe condition, indicating an easier defibration of woody xylem tissue in Sekizaisou. These results indicate that the delignification efficiency of Sekizaisou wood is higher than that of the other cultivars.

Many factors may contribute to the easier processing characteristics of Sekizaisou wood (Chanoca et al. 2020). In the case of typical hardwood species, such as poplar and *Eucalyptus*, S/G ratio in lignin is more directly correlated with alkaline pulping efficiency than is lignin content (Carrillo et al. 2018; del Rio et al. 2005; Francis et al. 2006; Guerra et al. 2008; Santos et al. 2011; Stewart et al. 2006). Increases in S/G ratio also contribute to delignification efficiency and improved pulp yield of woods derived from transgenic poplars overexpressing the ferulate 5-hydroxylase gene (Huntley et al. 2003). In contrast, an apparent negative correlation can be observed between lignin content of wood and pulp yield in other hardwood species, including *Artocarpus elasticus*, which belongs to the same family (Moraceae) as mulberry (*Morus*) (Istikowati et al. 2016; Santos et al. 2012; Yamada et al. 1992). Unlike other hardwoods with higher pulp yields, the S/G ratio in Sekizaisou wood, as determined by both thioacidolysis and nuclear magnetic resonance

spectroscopy, is significantly lower than those of other mulberry cultivars (Yamamoto et al. 2020). Thus, it is highly likely that the lower lignin content and the incorporation of hydroxycinnamaldehydes in Sekizaisou wood, as reported by Yamamoto et al. (2020), are causative to its easier delignification under soda pulping conditions.

As substitutes for the monolignols coniferyl and sinapyl alcohols, the incorporation of hydroxycinnamaldehydes into lignin generates arylglycerol-β-(hydroxycinnamaldehyde) ether substructures instead of arylglycerol β-aryl ethers. A carbonyl group conjugated with the aromatic ring in lignin is expected to favor the cleavage of the β-aryl ether bond, as seen in the case of the ring-conjugated sulfinyl group, which has an electron withdrawing effect similar to that of a carbonyl group (Ando et al. 2012). A conjugated hydroxycinnamaldehyde residue (linked 4-O-β to the unit being cleaved) renders it a far better leaving group in the cleavage of the β-ether units, as its phenolate anion produced under the alkaline conditions is more stable than the canonical non-conjugated phenolate. This might contribute, at least in part, to the accelerated delignification of lignin in Sekizaisou under alkaline conditions.

Lapierre et al. (1999) revealed that downregulation of *CAD* expression in transgenic poplars by antisense RNA technology substantially increased the frequency of free-phenolic units in lignin, accelerating delignification of the wood under alkaline pulping. Van Acker et al. (2017) also reported that *CAD1* suppression in poplars by RNA interference reduced lignin content and increased the frequency of free-phenolic groups in lignin. These changes may additionally contribute to the more favorable delignification characteristics of the poplar woods after an alkaline pretreatment. Although it is unknown whether these changes in lignin structure occur in Sekizaisou, similar alterations in phenolic metabolism, including substantial incorporation of sinapaldehyde into lignin, have been observed in Sekizaisou, just as in the *CAD1*-deficient transgenic poplars (Van Acker et al. 2017; Yamamoto et al. 2020).

3.5 Enzyme saccharification

Improved saccharification efficiency associated with changes in lignin content and composition has been reported in some woody species such as *Eucalyptus*, poplar, and pine (Chanoca et al. 2019). CAD suppression is also known to improve the saccharification efficiency in transgenic plants and mutants in alfalfa, poplar, and rice (Jackson et al. 2008; Martin et al. 2019; Van Acker et al. 2017). To further characterize Sekizaisou wood, the enzyme saccharification efficiency of pulverized wood from Sekizaisou and other mulberry cultivars was measured. Even without alkaline pretreatment (Figure 4), sugar release from Sekizaisou (23.1%) was significantly higher

than those of Kataneo (18.3%) and Chosenzairaishu (16.8%) (Figure 4). The released sugar levels in all cultivars were increased after the alkaline pretreatment and significant differences were detected in Sekizaisou compared to others except for Kinsou. Although the value of Sekizaisou after the pretreatment with 62.5 mM NaOH was comparable to that of Kinsou, the saccharification efficiency of Sekizaisou wood after the mild alkaline treatment (6.25 mM NaOH, Figure 4) was higher than that of Kinsou wood in which detected total monosaccharides was significantly higher than those in Sekizaisou (Table 1). Collectively, these results indicate that changes in lignin content and structure caused by the *CAD* gene deficiency in Sekizaisou facilitate not only the delignification of the wood during chemical pulping but also the sugar recovery upon enzymatic saccharification.

4. Conclusions

Stem lignin is easier to extract from the cell walls of the Sekizaisou cultivar than from those of the reference cultivars, due to lower lignin content and/or altered lignin structure in Sekizaisou. Sekizaisou, as well as lines derived from Sekizaisou that are homozygous for the mutation in the *CAD1* gene, have a great multiple-use potential as a biorefinery crop, in addition to their traditional use as a source for mulberry fruit, and as feed for silkworm and livestock. For a more extensive evaluation of the value of the *CAD1* mutation to the biorefinery performance of mulberry wood, further analyses with wood samples derived from a full-sib family that segregates for the mutant CAD-deficient phenotype will be required.

Author contributions:

TI, NT, SS, HS, N, and SH conducted experiments. NM, WB, JR, and SK analyzed data. TI, NT, NM, WB, JR, and SK wrote the manuscript. All the authors have accepted responsibility for the manuscript and approved the submission.

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Declaration of 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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Table and figure captions:

Table 1. Qualitative monomeric composition of polysaccharides in extractive-free cell walls prepared from branching stems of different cultivars after hydrolysis by sulfuric acid (Sakamoto et al. 2015). The results shown are means from three biological replicates (mg/g of cell wall) with standard deviations. * and ** represent significance differences from the values of Sekizaisou at $P < 0.05$ and $P < 0.01$, respectively (one-way ANOVA followed by Dunnett's test). [a] Total sugar represents the sum of all detected monosaccharides. Abbreviations: Ara, L-arabinose; Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; GlcA, D-glucuronic acid; Man, D-mannose; mGlcA, 4-*O*-methyl D-glucuronic acid; Rha, L-rhamnose; Xyl, D-xylose.

Table 2. Characteristics of pulp samples prepared from wood chips of five different mulberry cultivars under the two different alkaline conditions. The values are means from three independent technical replicates with standard deviations in brackets. [a] Residual lignin content in resultant wood pulp was measured by the Klason method. [b] Knots refer to uncooked dark aggregates of wood fibers detected after cooking and were counted based on the amount of wood chips used. ND, not detected. Superscripts [c], [d], and [e] represent significant differences from the values of Sekizaisou at $P < 0.1$, 0.05, and 0.01, respectively (one-way ANOVA followed by Dunnett's test).

Figure 1. Anatomical characteristics of Sekizaisou and a control mulberry, Nezumigaeshi. Debarked stems of Sekizaisou (a) and Nezumigaeshi (e) prepared from plants in winter. Transverse sections of stems (Sekizaisou: b, c, and d; Nezumigaeshi: f, g, and h) stained by toluidine blue (b and f), Wiesner (c and g), and Mäule (d and h) reagents. The figures shown in b-d and f-g are representatives of sections of three independent plants. Bars indicate 20 µm.

Figure 2. Density of mulberry woods prepared from 1-year-old stems. The values are means from three independent biological replicates with standard deviations as error bars. An asterisk indicates a significant difference from the value of Sekizaisou at $P < 0.01$ (one-way ANOVA followed by Dunnett's test).

Figure 3. Length and width of xylem fiber in stems of five different cultivars. Black and white bars indicate the length and the width, respectively. The values are means from three independent biological replicates with standard deviations as error bars. Different letters indicate statistically significant differences from one another as detected by Tukey's test at $P < 0.05$ or < 0.01.

Figure 4. Enzyme saccharification efficiency of wood powder prepared from Sekizaisou and other cultivars with (6.25 mM or 62.5 mM NaOH) and without alkaline pretreatment. The values are the means of three independent biological replicates. Error bars indicate the standard deviation. Different letters indicate statistically significant differences between the same treatment groups at $P < 0.01$ (one-way ANOVA followed by Dunnett's test or Tukey's test). ND, not determined.

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Table 1. Qualitative monomeric composition of polysaccharides in extractive-free cell walls prepared from branching stems of different cultivars after hydrolysis by sulfuric acid (Sakamoto et al. 2015). The results shown are means from three biological replicates (mg/g of cell wall) with standard deviations. * and ** represent significance differences from the values of Sekizaisou at $P < 0.05$ and $P < 0.01$, respectively (one-way ANOVA followed by Dunnett's test).

Cultivars	Glc	Xyl	Rha	GalA	mGlcA
Sekizaisou	283 ± 9.7	162 ± 5.9	49.8 ± 1.4	21.1 ± 1.6	16.5 ± 2.3
Kataneo	310 ± 28	181 ± 14*	56.3 ± 5.7	24.5 ± 2.8	18.9 ± 1.7
Chosenzairaishu	354 ± 6.3**	175 ± 1.6	54.3 ± 3.4	20.9 ± 1.6	20.2 ± 0.5*
Kinsou	346 ± 2.1**	175 ± 2.1	57.5 ± 4.4*	22.8 ± 2.6	22.3 ± 1.3**
Nezumigaeshi	292 ± 8.7	139 ± 10*	51.3 ± 2.2	19.3 ± 1.5	15.9 ± 1.9

Cultivars	Man	Gal	Ara	GlcA	Total sugar[a]
Sekizaisou	11.6 ± 0.9	9.18 ± 0.38	3.65 ± 0.1	1.32 ± 0.09	558 ± 18
Kataneo	8.91 ± 1.2*	9.44 ± 1.52	3.78 ± 0.87	1.72 ± 0.31*	614 ± 49*
Chosenzairaishu	12.0 ± 0.7	8.81 ± 0.97	3.05 ± 0.61	1.66 ± 0.11	650 ± 4.3**
Kinsou	11.8 ± 1.1	9.27 ± 1.5	3.04 ± 0.69	1.38 ± 0.21	649 ± 9.2**
Nezumigaeshi	10.8 ± 1.1	9.72 ± 0.66	2.51 ± 0.14	1.47 ± 0.07	542 ± 22

[a] Total sugar represents the sum of all detected monosaccharides. Abbreviations: Ara, L-arabinose; Gal, D-galactose; GalA, D-galacturonic acid; Glc, D-glucose; GlcA, D-glucuronic acid; Man, D-mannose; mGlcA, 4-O-methyl D-glucuronic acid; Rha, L-rhamnose; Xyl, D-xylose.

Table 2. Characteristics of pulp samples prepared from wood chips of five different mulberry cultivars under the two different alkaline conditions. The values are means from three independent technical replicates with standard deviations in brackets.

Cultivars	30% NaOH			25% NaOH		
	Pulp yield (%)	Residual lignin (%)[a]	Knot (%)[b]	Pulp yield (%)	Residual lignin (%)[a]	Knot (%)[b]
Sekizaisou	46.8 (0.5)	5.3 (0.2)	0.01 (0.02)	49.8 (0.3)	7.0 (0.8)	ND
Kataneo	45.4 (1.7)	6.7 (0.6)[d]	0.50 (0.47)	42.3 (5.3)[d]	8.9 (1.2)[d]	8.4 (6.0)
Chosenzairaishu	43.1 (0.6)[d]	6.8 (1.1)[d]	0.89 (0.78)	42.4 (4.9)[d]	9.4 (0.9)[e]	8.8 (6.2)
Kinsou	44.8 (1.1)[c]	6.2 (0.6)	2.2 (1.3)[d]	39.5 (1.7)[e]	8.4 (0.5)[c]	12 (1.6)
Nezumigaeshi	44.8 (0.9)[c]	7.3 (0.5)[e]	1.3 (0.81)	41.2 (2.1)[d]	11 (0.5)[e]	10 (0.7)

[a] Residual lignin content in resultant wood pulp was measured by the Klason method. [b] Knots refer to uncooked dark aggregates of wood fibers detected after cooking and were counted based on the amount of wood chips used. ND, not detected. Superscripts [c], [d], and [e] represent significant differences from the values of Sekizaisou at $P < 0.1$, 0.05, and 0.01, respectively (one-way ANOVA followed by Dunnett's test).

Figure 1.

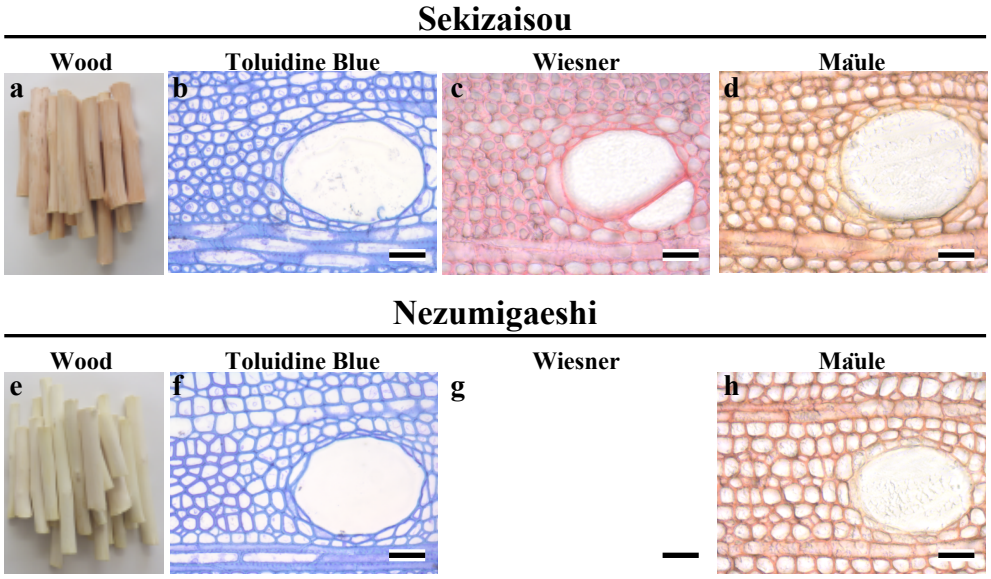

Sekizaisou
Wood
Toluidine Blue
Wiesner
Mäule
a
b
c
d
Nezumigaeshi
Wood
Toluidine Blue
Wiesner
Mäule
e
f
g
h

Figure 2.

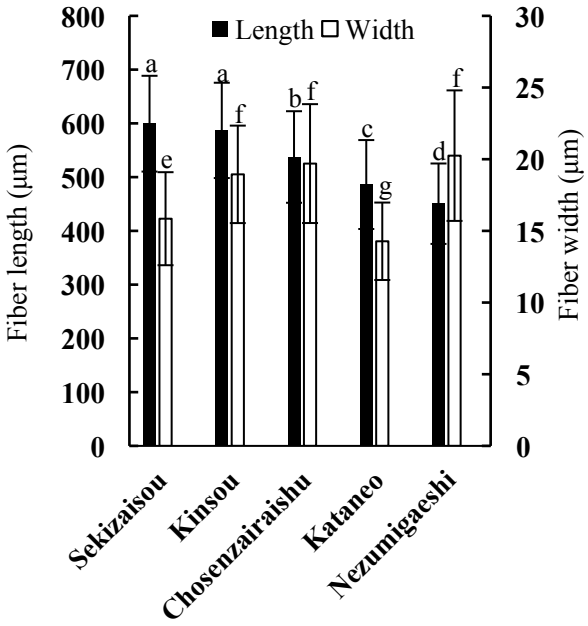

Length
Width
Fiber length (μm)
Fiber width (μm)
800
700
600
500
400
300
200
100
0
30
25
20
15
10
5
0
a
e
a
f
b
f
c
g
d
f
Sekizaisou
Kinsou
Chosenzairaishu
Kataneo
Nezumigaeshi

Figure 3.

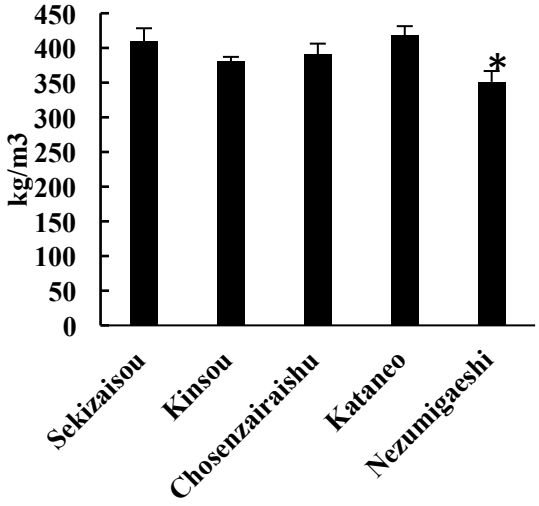
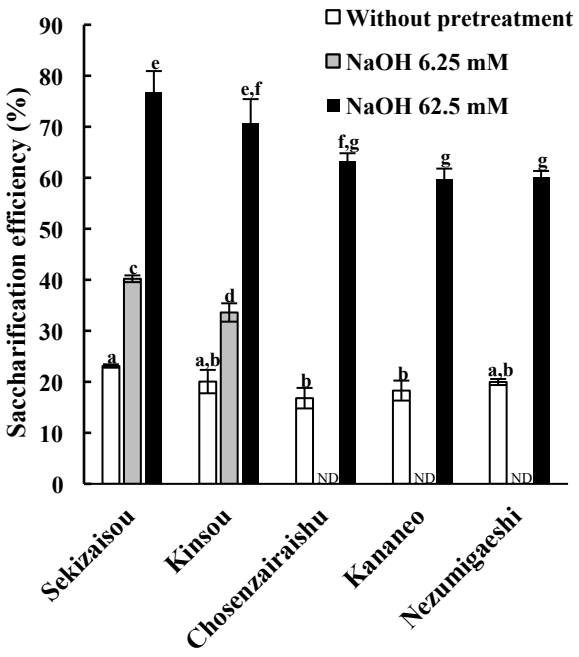

kg/m3
450
400
350
300
250
200
150
100
50
0
*
Sekizaisou
Kinsou
Chosenzairaishu
Kataneo
Nezumigaeshi

Figure 4.


Without pretreatment
NaOH 6.25 mM
NaOH 62.5 mM
Saccharification efficiency (%)
90
80
70
60
50
40
30
20
10
0
a
c
e
a,b
d
e,f
b
f,g
b
g
a,b
g
ND
ND
ND
Sekizaisou
Kinsou
Chosenzairaishu
Kananeo
Nezumigaeshi