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Simultaneous manipulation of lignin structure and secondary cell wall formation in transgenic poplar

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Abstract

Increasing the wood density to a preferred range contributes to upgrading the value of wood as raw material. Lignin manipulation can also improve wood quality. This study attempted to accelerate secondary cell wall formation in transgenic poplar with an altered lignin structure. To achieve this, *OsSWN1*, a rice master switch for secondary cell wall formation in fiber cells, was overexpressed in poplar plants in which monolignol biosynthesis was suppressed by the expression of an RNA-interference construct targeted to the gene for cinnamyl alcohol dehydrogenase 1. The generated transgenic poplars successfully overexpressed the chimeric *OsSWN1* construct and monolignol biosynthesis remained altered in these plants. Secondary cell wall thickness was increased in the transgenic plants and their wood density was higher compared to the background line. No difference in lignin content was observed, except in one transgenic line. High saccharification characteristics observed in the background line were preserved in the transgenic line with lower *OsSWN1* overexpression, but higher *OsSWN1* overexpression had a slight negative impact on enzyme saccharification. Our data suggested that fine-tuning of accelerated deposition of the secondary cell wall combined with alteration of monolignol biosynthesis should improve the lignocellulose quality for conventional and future biorefinery uses.

Keywords: Genetic engineering, Poplar, Transcription factor, Wood density, Xylem

Introduction

Lignin is a major chemical component of cell walls in terrestrial plant biomasses. This phenolic polymer is mainly built from three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, which are biosynthesized from phenylalanine or tyrosine via the cinnamate/monolignol pathway [1, 2]. In the last step of monolignol biosynthesis in the cytosol, *p*-hydroxycinnamaldehydes are reduced by cinnamyl alcohol dehydrogenase (CAD) to form monolignols [3]. After monolignols are transported to the cell wall, they are radicalized by phenoloxidases such as

laccase and peroxidase, and formed *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units in the lignin polymer [2]. In addition to monolignols, *p*-hydroxycinnamaldehydes and other phenolics are incorporated into lignin as minor units [4].

Lignin gives cell walls their physical strength to support upward plant growth, facilitates water transport in plants, prevents microbial attacks, and protects plants from UV irradiation. Although lignin exhibits various essential roles for plant growth and development, it is highly resistant to chemical and biochemical pretreatment for the efficient utilization of the plant cell wall as lignocellulosic biomass [5]. Even for partial removal of lignin from the lignocellulosic biomass, especially woody biomass, material processing generally requires a substantial amount of energy and chemical consumption.

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Decreasing the lignin content and changing the lignin structure in plant cell walls by genetic engineering via the manipulation of lignin biosynthesis genes can reduce lignin resistance in pretreatment processes [5]. Extensive studies have been performed in this area using *Populus* as a model tree species [6]. For example, suppression of *p*-coumaroyl-CoA 3'-hydroxylase and cinnamoyl-CoA oxidoreductase in transgenic poplars induce lignin reduction and/or structural changes [7, 8]. These modifications result in improved enzyme saccharification efficiency in the wood tissue of transgenic plants.

CAD downregulation via expression of antisense and sense RNA constructs also induces structural modification of lignin [9, 10]. In addition, CAD suppression by a hairpin RNA construct and a nonsense mutation of CAD lead to reduced lignin content with an altered structure [11–13]. CAD reductions in woody species result in the incorporation of substantial amounts of *p*-hydroxycinnamaldehyde units, especially sinapaldehyde units, into lignin [11, 12, 14]. CAD suppression also results in an increase in the number of free phenolic groups in the lignin, which enhance the reactivity of lignin under alkaline conditions. This improves lignin degradation efficiency under alkaline conditions, and hence, improves saccharification efficiency after alkaline pretreatment [10, 11, 15]. Although lignin modification by genetic engineering targeting lignin biosynthetic genes can occasionally lead to abnormal plant growth and impose a yield penalty on plant biomass, no severe negative impact on biomass yield has been reported in woody plants showing CAD downregulation under greenhouse cultivation and in field trials [10, 11].

In addition to improved lignin extractability, increasing wood density within a preferred range contributes, at least in part, to upgrading wood value as a raw material for biorefinery usage. Greater wood density means higher biomass production per unit of land and a larger amount of the biomass-derived product from a reactor per unit of time if the plant growth and product yield are independent of the density.

One of the options for improving wood density by genetic engineering is overexpressing the transcriptional regulator of secondary cell wall (SCW) biosynthesis. Sakamoto et al. [16] showed that expression of the rice secondary wall NAC (*No apical meristem, Arabidopsis* transcription activation factor, and *Cup-shaped cotyledon*) domain protein 1 (*OsSWN1*) in transgenic poplars resulted in increased cell wall thickness in fiber cells and increased wood density. This protein is the ortholog of the master regulator of SCW formation in *Arabidopsis*, NAC secondary wall thickening promoting factor 3/SCW associated NAC domain protein 1 (*NST3/SND1*) [17–21]. The growth of transgenic poplars expressing

OsSWN1 under the control of the *NST3* promoter is indistinguishable from that of wild-type plants under soil cultivation [16]. *OsSWN1* overexpression in poplars induces structural modification of lignin but no significant change in lignin content has been observed.

In the present study, we evaluated the synergetic effect of suppressed lignin biosynthesis and enhanced SCW deposition in poplar with a simultaneous downregulation of *CAD1* and overexpression of *OsSWN1*.

Materials and methods

Poplar transformation

A chimeric *OsSWN1* construct under the control of the *Arabidopsis NST3* promoter [16] was introduced into hpCAD19 transgenic poplar line (*Populus tremula* × *Populus alba* clone INRA 717-1B4) [11] in which *CAD1* activity was suppressed by the cauliflower mosaic virus 35S promoter-driven hairpin RNAi strategy. Tissue-preferential expression of the *Arabidopsis NST3* promoter in secondary xylem and phloem fibers in stems, in addition to ray parenchyma and vessel elements, were visualized by a histochemical promoter-reporter assay of the transgenic poplar plants [22]. Conventional genetic transformation via an *Agrobacterium*-mediated procedure was performed following the method described by Leplé et al. [23]. Hygromycin (10 mg/L) was added to the culture medium for the selection of transformed cells and subsequent regenerated plants. The resultant transgenic lines were coppiced by cutting in the medium with hygromycin and cultivated in soil for further use. The cultivation was performed in a culture room at 25 °C under long-day (16-h light:8-h dark) conditions.

Semiquantitative and quantitative reverse transcription-polymerase chain reaction

Stem tissues were collected from the 11th and 12th internodes of 4-month-old plants and immediately frozen in liquid nitrogen. Isolation of total RNA was performed using TRIzol (Thermo Fisher Scientific K.K., Tokyo). After DNase treatment of the resultant total RNA, 1st strand cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics K.K., Tokyo) for semiquantitative RT-PCR. DNA derived from *CAD1* transcripts was amplified using GoTaq DNA Polymerase (Promega K.K., Tokyo). The ubiquitin transcript was also amplified as a positive control. PCR was performed in a cycling condition as follows: 3 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C with a final step at 72 °C for 5 min. Reaction mixtures with the amplified products (10 and 5 µl for *CAD1* and ubiquitin, respectively) were separated by 1% agarose gel electrophoresis. Quantitative RT-PCR was performed as described

by Takata and Taniguchi [24]. First-strand cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Real-time PCR was carried out using a StepOnePlus Real-Time PCR System with PowerUp SYBR Green PCR Master Mix (Thermo Fisher Scientific). Gene expression levels were normalized to those of 18S ribosomal RNA. RNA samples were assayed in triplicate with three biological replicates. The gene-specific primers for PCR are listed in Additional file 1: Table S1.

Histochemical analysis

Stem segments were sampled from the 5th and 6th internodes of each transgenic plant. Tissue sections (10- μ m thick) were cut using a microtome (model MTH-1, Nippon Medical & Chemical Instruments Co., Ltd., Osaka). To measure cell wall thickness by image analysis, the cross sections were stained with 1% safranin solution and images were obtained using a microscope (model BZ-X810, Keyence Corporation, Osaka). Cell wall thickness of over 400 xylem cells excluding vessel elements for each transgenic line were analyzed semi-automatically using binarized images obtained from safranin-stained sections using the Local Thickness plugin of ImageJ 2.0.0 [25, 26]. Lignin autofluorescence of an unstained thin section was monitored using a microscope equipped with a DAPI filter set (type OP-87762, Keyence Corporation; excitation filter, 340–380 nm; dichroic mirror, 400 nm; emission filter, 435–485 nm long pass).

Lignin content and composition

Lignin characterization was performed with stem (4 months old) after bark peeling. After the air drying for 1 month at room temperature, a top part (approximately 3 cm in length) of the dried stem wood was removed for measurement of density. Remaining part of the wood was pulverized with a Multi-Beads Shocker (MB1200C, Yasui Kikai Corporation, Osaka) until the wood powder passed through a 150- μ m screen. Cell wall residue (CWR) was prepared from the powder by sequential extraction using 50 mM NaCl solution, hot water, methanol, 99.5% ethanol, a mixture of ethanol and toluene (1:2, v/v), and acetone. Lignin content in CWR (ca. 5 mg) was measured using an acetyl bromide protocol as described previously [25]. Monomeric composition of lignin in CWR was analyzed by thioacidolysis. Conventional thioacidolysis products were quantified by gas chromatography equipped with a flame-ionization detector after trimethylsilylation [14, 25, 27]. For lignin content and composition analyses, three biological replicates were analyzed for each plant line.

Wood density

Density of the air-dried wood pieces described above was calculated from volume and mass of each piece. Volume of the wood piece was measured on Archimedes' principle in a beaker filled with Milli-Q water on a conventional laboratory balance. Density of gently packed CWR was measured using a PCR tube (0.2 mL volume). Masses of same volume of Milli-Q water and packed CWRs in the tube were measured on the balance and then used to calculate the density by dividing the mass by the volume. Preparation of CWR was described above.

Enzyme saccharification

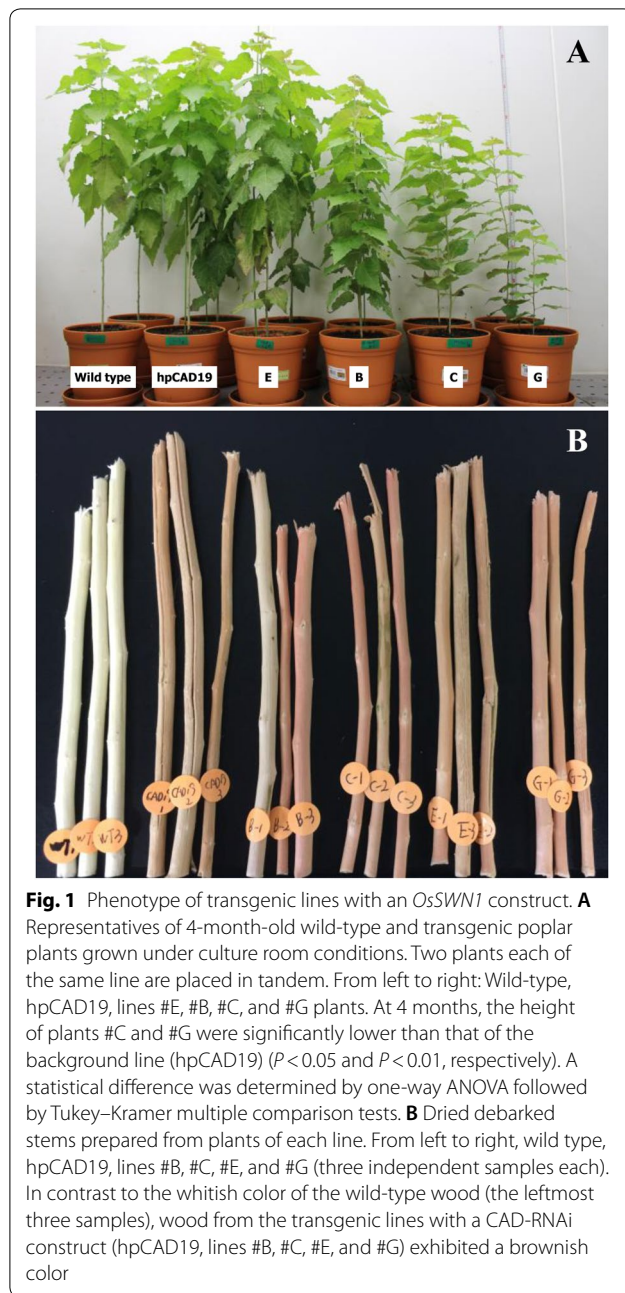
Saccharification efficiency was monitored after diluted alkali pretreatment. CWR (ca. 10 mg) was mixed with 300 μ L of 100 mM NaOH solution and heated at 120 °C for 1 h. After cooling to room temperature, the solution was neutralized with diluted hydrochloric acid. Saccharification was then carried out in 50 mM citrate buffer (pH 4.8) with Celluclast (Sigma-Aldrich C2730) and Cellobiase (Sigma-Aldrich C6105) at 50 °C for 14 h. Sugars released from the samples were quantified by 3, 5-dinitrosalicylic acid assay using glucose as a standard [28]. Saccharification efficiency was calculated based on the amounts of released sugar and CWR without pretreatment. Three biological replicates were analyzed for each plant line.

Results and discussion

Transgenic poplar generation and *OsSWN1* expression

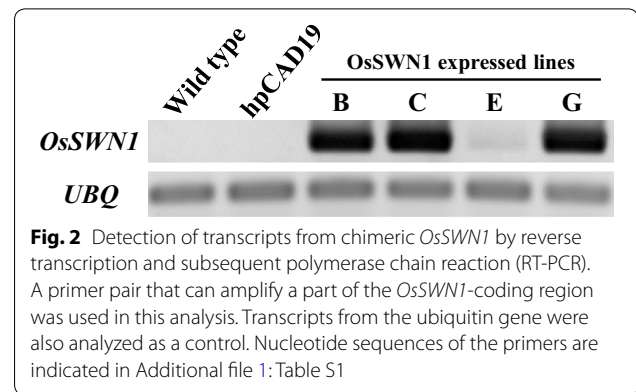
The tissue-preferential pattern of *NST3* expression met our objective, i.e., SCW formation was promoted in poplar stem xylem by the chimeric overexpression of *OsSWN1* [22]. After the genetic transformation of the hpCAD19 line via an *Agrobacterium*-mediated transformation procedure, we obtained over 10 independent transgenic lines with the chimeric *OsSWN1* construct. We selected four independent *OsSWN1*-expressing lines (lines #B, #C, #E, and #G) for further analysis based on their growth stability during successive propagation by in vitro cuttings in the culture medium. Although they could be maintained by sequential in vitro propagation for over a year, cuttings from lines #C and #G were relatively difficult to root compared to the wild-type and other transgenic lines (data not shown).

Figure 1A shows each transgenic line placed in two rows in a culture room after soil cultivation for 4 months. Although the height of line #B (60.5 ± 13.7 cm) was slightly lower than that of wild-type plants (79.3 ± 3.9 cm), no statistical differences were detected at $P < 0.01$ level among the wild-type and hpCAD-19 (85.5 ± 7.8 cm) lines, and the plant lines #B



and #E (76.7 ± 3.8 cm). In contrast, growth retardation was observed in lines #C (54.0 ± 7.3 cm) and #G (33.5 ± 17.7 cm). This might be due, in part at least, to the introduction of the chimeric *OsSWN1* construct into the plants.

To validate chimeric *OsSWN1* expression, semiquantitative RT-PCR was performed using total RNA isolated from each plant. Expression was detected in the four *OsSWN1* lines, but the *OsSWN1* level in line #E was apparently lower than that of the other *OsSWN1* lines



(Fig. 2). These results indicated that *OsSWN1* expression level varied among the different plant lines though difference in the expression level among the lines #B, #C, and #G was unclear in our present data. No amplified DNA derived from the transgene was observed in the wild-type and hpCAD19 plants.

Endogenous *CAD1* expression

As reported previously, heterologous *OsSWN1* expression can induce *CAD* (*CAD-D*) expression in transgenic *Arabidopsis* [16] and *CAD1* expression in transgenic poplar [25]. These results suggest that simultaneous expression of chimeric *OsSWN1* and *CAD1*-RNAi transgenes is expected to result in a conflicting response on endogenous *CAD1* expression in the present lines. Thus, we checked whether sufficient *CAD1* suppression could still be achieved in the four transgenic *OsSWN1* lines. As indicated in Additional file 1: Figure S1, *CAD1* expression remained depressed in all four *OsSWN1* lines and no significant differences were detected compared to the hpCAD19 line, although the suppression levels varied slightly among the tested plants.

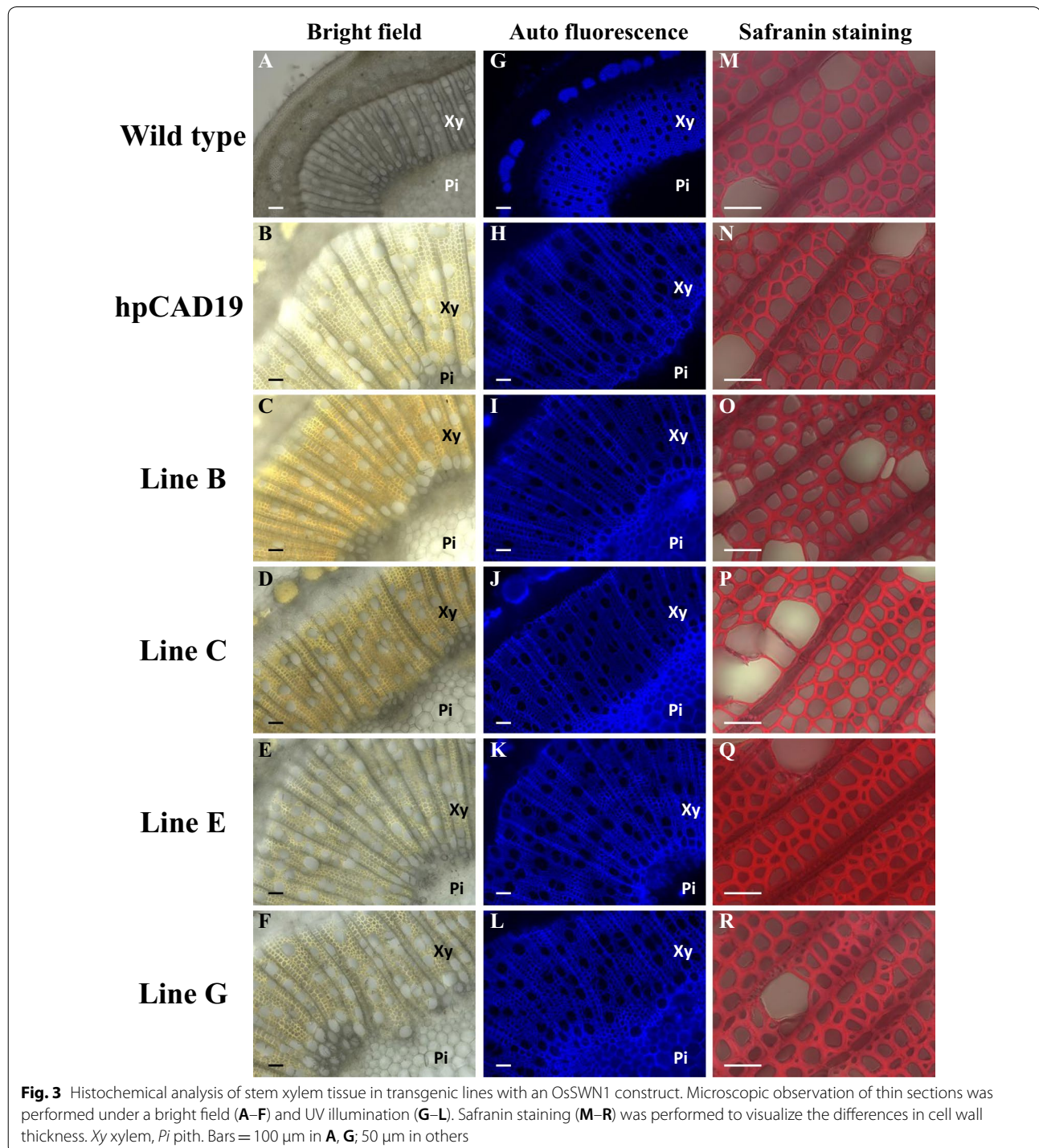
Unlike wild-type poplar, the CAD-deficient hpCAD19 line exhibited red coloration in its xylem tissue that turned to brown after drying (Fig. 1B). This coloration is a known indicator of *CAD* deficiency in various plant species [14]. As indicated in Fig. 1B, the four *OsSWN1* lines also showed brown xylem tissue coloration, indicating that endogenous *CAD1* expression was still efficiently suppressed in the transgenic poplars. This supports the findings of the real-time PCR analysis shown in Additional file 1: Figure S1.

Histochemical analysis of stem tissues

For further characterization of the effect of chimeric *OsSWN1* expression on accelerating SCW formation, we analyzed lignin deposition and cell wall thickness in stems using thin sections of stem tissues. At lower

magnification (10× for wild type and 20x for other lines) under bright field observation, no apparent histochemical differences were observed between the wild-type, hpCAD19, and *OsSWN1*-expressing lines (Fig. 3A–F). In contrast, microscopic observation of autofluorescence from plant tissues differed among the tested lines. No

autofluorescence occurred in the pith parenchyma tissues of the wild-type (Fig. 3G), hpCAD19 (Fig. 3H), and line #E (Fig. 3K) plants. In contrast, strong autofluorescence was detected in the three *OsSWN1* lines (#B, #C, and #G; Fig. 3I, J, L) in which the *OsSWN1* expression was apparently higher than the line #E, indicating that



lignification was accelerated in the pith tissue, where less lignification generally occurs [29]. Accelerated pith lignification was previously reported in *OsSWNI*-expressing poplars generated in a wild-type background [16].

Under microscopic observation of safranin-stained sections at high magnification, cell wall thickness of xylem tissue in hpCAD19 plants seemed to be thinner than that of the wild-type plants, though the differences were not remarkable (Fig. 3M, N). In contrast, the cell wall thickness in lines #B and #G (Fig. 3O, R) were higher than those of hpCAD19 and wild-type plants. These results suggested that *OsSWNI* expression induced enhanced cell wall formation in transgenic plants.

Quantitative characterization of accelerated cell wall formation by imaging analysis

To confirm the enhanced cell wall thickness in *OsSWNI* lines, an image analysis of wood fiber cells was performed based on microscopic imaging of safranin-stained thin sections as described in previous studies [16, 25]. Analysis showed that the average cell wall thickness of fibers in the hpCAD19 line ($1.63 \pm 0.25 \mu\text{m}$, $N=1405$) was slightly but significantly lower than that of the wild-type line ($1.81 \pm 0.27 \mu\text{m}$, $N=934$) (Fig. 4). This result suggested that lignin modification by *CAD1* suppression accompanied reduced cell wall thickness in the fibers. In contrast, the cell wall thickness of the *OsSWNI*-expressing lines was significantly higher (3.7% higher in #E, 17% higher in #C, and 29% higher in #B and #G) than that of the

wild-type line (Fig. 4). Our data indicated that *OsSWNI* expression promoted cell wall thickening even under *CAD1* suppression and restored the reduced thickness observed in the hpCAD19 line.

Moisture content in fresh wood

Generally, enhanced wall thickness is expected to induce increased wood density. Since the moisture content of fresh wood has a roughly negative correlation with its density, we calculated the moisture content ($[\text{fresh weight} - \text{dry weight}]/\text{fresh weight}$) of debarked wood prepared from the stems of 4-month-old plants immediately after harvest and subsequent air-drying for 1 month under room conditions. Although wood moisture could not be completely removed by this procedure, moisture content of the whole stem samples could be estimated. As shown in Additional file 1: Figure S2, the moisture content of the wild type and hpCAD19 line were on the same level. In contrast, those in lines #B and #C were significantly reduced by 14% and 15%, respectively, compared to the wild type. Compared to hpCAD19, the moisture content in lines #B, #C, and #G were also significantly reduced by 15%, 16%, and 7%, respectively. In contrast, the moisture content in line #E (in which the *OsSWNI* expression was relatively low) was comparable to those of the wild type and the hpCAD19 line. Collectively, our results suggested that chimeric *OsSWNI* expression led to decreased fresh moisture content in the debarked stem. The decrease might be due to decrease in intracellular space occurring along with accelerated thickening of SCW in xylem cells as previously reported in other transgenic poplars harboring *OsSWNI* [25]. The enhanced SCW deposition also led to increase in wood density as discussed in the next section.

Stem wood density

The wood density of the top 3-cm part of harvested transgenic stems was determined based on Archimedes' principle (black bars in Fig. 5). The averaged values of three biological samples from lines #B and #C were 19% and 24% higher than that of the wild-type plants, respectively. In contrast, the wood density of hpCAD19 plants was 15% lower than that of the wild-type plants. Although reduced wood density in hpCAD19 plants has not been previously reported, wood density in hpCAD24 line with the same *CAD1*-RNAi construct was significantly lower than that of its wild-type counterparts [30]. In contrast to the higher level of cell wall thickness enhancement detected in line #G (Fig. 4), no difference in wood density was observed compared to that of the wild-type plant. This may partially be due to the differences in stem parts used in these analyses and the growth retardation of line #G.

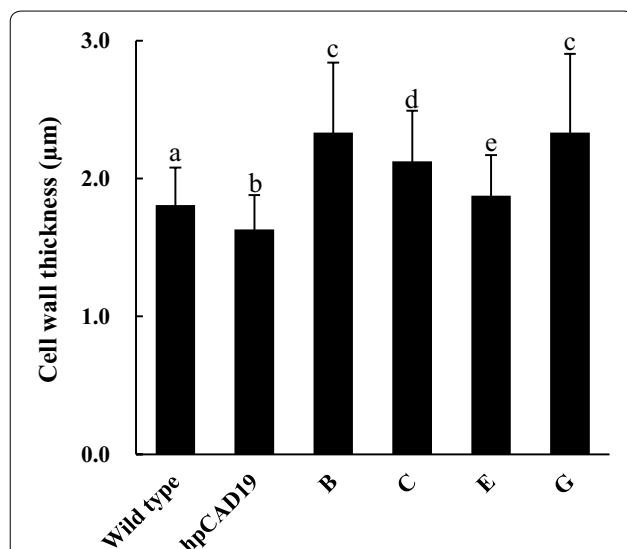
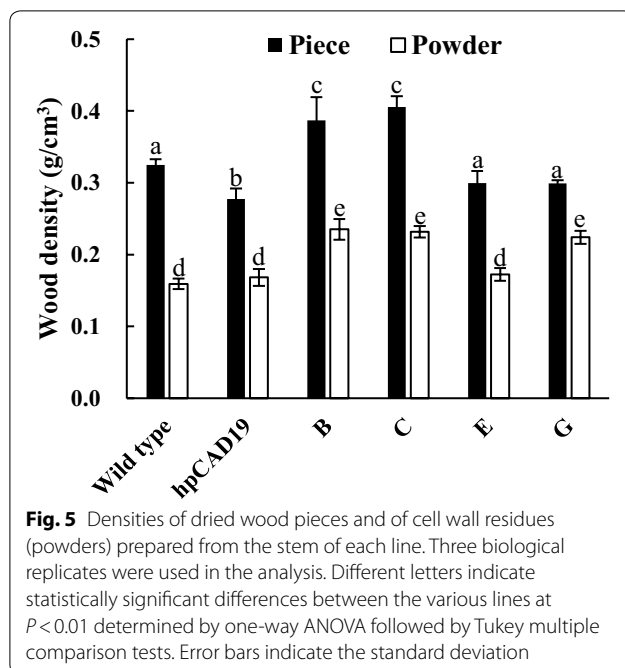


Fig. 4 Cell wall thickness of xylem cells measured by imaging analysis. Different letters indicate statistically significant differences between the various lines at $P < 0.01$. A statistical difference was determined by one-way ANOVA followed by Tukey–Kramer multiple comparison tests. Error bars indicate the standard deviation



Particle density (packed density) was also measured using CWR prepared from stem wood of each line (white bars in Fig. 5). The values were significantly increased in lines #B, #C, and #G which exhibited higher *OsSWNI* overexpression compared to wild type, hpCAD19, and line #E with lower expression of *OsSWNI*. Collectively, present results indicate that *OsSWNI* overexpression contributes to increase in the stem wood density of the plants with an hpCAD19 background, as reported previously in a wild-type background [16, 25].

Lignin characterization

Lignin content in CWR, as determined by an acetyl bromide protocol, showed no significant differences between the wild-type and the hpCAD19 lines (Additional file 1: Figure S3). Similarly, the lignin contents of lines #C, #E, and #G did not significantly differ from that of their host plant, line hpCAD19. In contrast, that of line #B was significantly increased by 14% compared to the hpCAD19 line. This discrepancy observed in the *OsSWNI* lines might be due to the expression level of the chimeric transcription factor in each line or the position effect of the gene transfer site in the chromosome.

In addition to the lignin content, the monomeric composition of lignin was also determined by thioacidolysis (Additional file 1: Figure S4). Detected levels of total thioacidolysis monomers (G plus and S monomers) in the hpCAD19 line were lower than those of wild-type plants, as reported previously [11]. The syringyl to guaiacyl (S/G) ratio of the hpCAD19 line (1.40) was relatively lower than

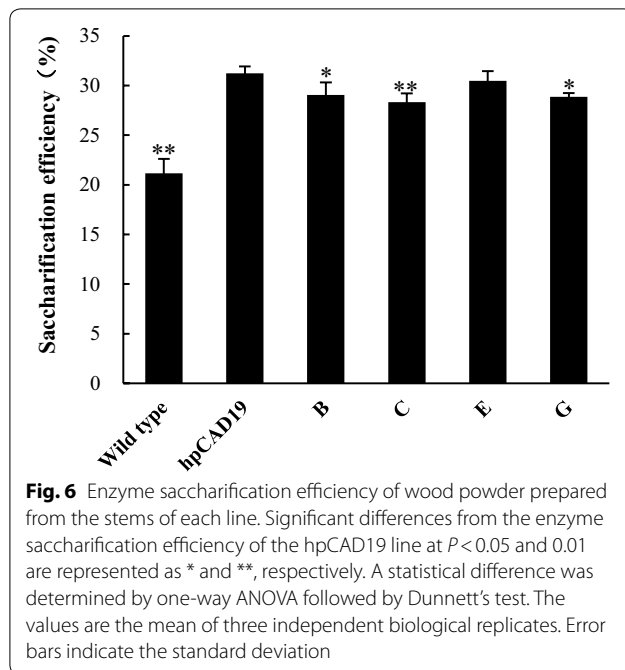
that of the wild-type plants (1.66, $P = 0.074$). Compared to the hpCAD19 plants, the level of the sum of the two monomers was increased in the four *OsSWNI* expressing lines but no significant differences were detected. In contrast, the G monomer level in lines #B, #C, and #G (which had higher *OsSWNI* expression) was significantly higher than that in the hpCAD19 plants. This change led to a significant reduction of the S/G ratio in these three lines (0.79, 0.88, and 0.98 for the #B, #C, and #G lines, respectively) compared to the hpCAD19 line. In line #E, which displayed relatively low *OsSWNI* expression, the S/G ratio (1.29) was comparable to that of the hpCAD19 line. These results suggested that chimeric *OsSWNI* expression in plants with an hpCAD19 background were more effective in changing lignin composition than lignin content, as observed in previous studies studying the effects of *OsSWNI* expression in a wild-type background [25].

As indicated in Additional file 1: Figure S1, remained *CAD1* suppression in the four *OsSWNI* lines could be confirmed by real-time PCR. The reduction of *CAD1* expression in poplar as well as other plant species known to lead incorporation of coniferaldehyde and sinapaldehyde into the lignin [11]. The incorporation could be detected as release of indene derivatives by thioacidolysis of lignin. Additional file 1: Figure S5 shows the indene release from the four *OsSWNI* lines as the hpCAD19 line. In contrast, no release from the wild-type plants could be observed. These results indicate that substantial amount of the hydroxycinnamaldehydes remains to be incorporated into lignin under overexpression of chimeric *OsSWNI*.

Saccharification efficiency

Lignin modification via *CAD1* suppression improves delignification efficiency under alkaline conditions [15] and the subsequent saccharification of CWRs [11]. Saccharification efficiency was measured after a simple alkaline pretreatment to examine the effect of *OsSWNI* expression-induced enhancement of cell wall thickness.

As in the previous study [11], CWR of the hpCAD19 line exhibited high saccharification efficiency compared to wild-type plants (Fig. 6). Although the saccharification efficiency in the *OsSWNI*-expression lines was still higher than that of the wild-type line, the efficiencies of the #B, #C, and #G lines were slightly but significantly lower than that of the hpCAD19 line. In contrast, no significant difference was observed between the hpCAD19 and line #E plants. These results indicated that excessive overexpression of chimeric *OsSWNI* created a trade-off between enhanced cell wall thickness and improved saccharification efficiency by lignin modification; however,



the improved characteristic of easy delignification by CAD1 suppression remained after cell wall thickness was enhanced by chimeric *OsSWN1* overexpression.

Conclusions

Overexpression of the *OsSWN1* transcription factor accelerated SCW deposition in transgenic poplar (hpCAD19), in which the last step of monolignol biosynthesis was suppressed and lignin structure was altered. High overexpression of chimeric *OsSWN1* led to an additional modification of lignin structure in the background line and had a slight negative impact on the enzyme saccharification efficiency of the CWR. In order to maximize the synergetic effect of lignin inhibition by *CAD1* suppression and enhanced cell wall deposition by *OsSWN1* overexpression for efficient lignocellulose production, an in-depth evaluation of the growth retardation mechanism observed in the resultant lines, in addition to the qualitative and quantitative regulation of lignin biosynthesis using the chimeric gene, should be a focus of future studies.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s10086-020-01902-2>.

Additional file 1: Table S1. Nucleotide sequences of primers used in RT-PCR and real-time PCR. **Figure S1.** *CAD1* expression analysis by real-time PCR. *CAD1* expression in wild-type plants and transgenic lines were monitored by real-time PCR using *CAD1*-specific primers. The values were expressed after normalization with the expression of 18S ribosomal

RNA gene in each plant. Data were obtained from three biological replicates. Error bars indicate the standard deviation. Different letters indicate statistically significant differences between the wild-type and the transgenic lines at $P < 0.01$. A statistical difference was determined by one-way ANOVA followed by Dunnett's test. **Figure S2.** Moisture content of debarked fresh stems. The value was calculated based on weight of debarked stem wood immediately after harvest compared to air-dried wood. A statistical difference was determined by one-way ANOVA followed by Tukey multiple comparison tests. Significant differences from hpCAD19 line values at $P < 0.05$ and 0.01 are represented as * and **, respectively. Error bars indicate the standard deviation. **Figure S3.** Lignin content in stem wood prepared from different lines. A significant increase in value was only detected in line #B compared to the wild-type line ($P < 0.05$) by one-way ANOVA followed by Dunnett's test. Error bars indicate the standard deviation. **Figure S4.** Monomeric composition of lignin evaluated by thioacidolysis and subsequent gas chromatography. Conventional thioacidolysis products with guaiacyl (G) and syringyl (S) nuclei were quantified by gas chromatography equipped with a flame-ionization detector after trimethylsilylation. Each value is expressed as a relative level of the amount detected in the hpCAD19 line. Significant differences from hpCAD19 line values at $P < 0.05$ and 0.01 are represented as * and **, respectively, using one-way ANOVA followed by Dunnett's test. The values are the mean of three independent biological replicates. Error bars indicate the standard deviation. **Figure S5.** Detection of indene compounds released by thioacidolysis of lignins. Thioacidolysis-released indenones (1_{G1} , 1_{S1} , and 1_{S2}), which are indicative of the incorporation of coniferaldehyde and sinapaldehyde into the lignin in CAD suppressed plants, could be detected in the transgenic lines but not in the wild-type plant by GC/MS analysis. Peaks labeled 1_{G1} , 1_{S1} , and 1_{S2} correspond to the indenones with different structures. Detection of the compounds indicates CAD expression is still suppressed in the plants with *OsSWN1* as in the hpCAD19 line.

Abbreviations

CAD: Cinnamyl alcohol dehydrogenase; CWR: Cell wall residue; NAC domain protein: No apical meristem, Arabidopsis transcription activation factor, and cup-shaped cotyledon domain protein; NST3: NAC secondary wall thickening promoting factor 3; *OsSWN1*: Rice secondary wall NAC domain protein 1; SCW: Secondary cell wall; SND1: SCW-associated NAC domain protein 1.

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Authors' contributions

Na, Nu, SW, NT, SS, MY, and MU performed most of the experiments. NT, AD, GP, NM, and SK designed the study and interpreted the data. Na, NT, AD, GP, TT, NM, and SK wrote the manuscript. NT, TT, NM, and SK managed the project. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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