Tetraploidization promotes radial stem growth in poplars

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Abstract Somatic polyploidization often increases cell and organ size, thereby contributing to plant biomass production. However, as most woody plants do not undergo polyploidization, explaining the polyploidization effect on organ growth in trees remains difficult. Here we developed a new method to generate tetraploid lines in poplars through colchicine treatment of lateral buds. We found that tetraploidization induced cell enlargement in the stem, suggesting that polyploidization can increase cell size in woody plants that cannot induce polyploidization in normal development. Greenhouse growth analysis revealed that radial growth was enhanced in the basal stem of tetraploids, whereas longitudinal growth was retarded, producing the same amount of stem biomass as diploids. Woody biomass characteristics were also comparable in terms of wood substance density, saccharification efficiency, and cell wall profiling. Our results reveal tetraploidization as an effective strategy for improving woody biomass production when combined with technologies that promote longitudinal stem growth by enhancing metabolite production and/or transport.

Key words: polyploidization, poplar, tetraploid, woody biomass.

Introduction

Polyploids are organisms that have more than two paired sets of chromosomes. Since the discovery of Oenothera gigas, a giant mutant variant of evening primrose Oenothera lamarckiana, numerous studies have shown that polyploidy in plants often increases cell and organ size, referred to as the "gigas" effect (De Vries 1905; Lutz 1907; Sattler et al. 2016; Song and Chen 2015). More than 70% of flowering plants are considered polyploids (Song and Chen 2015), including cultivated plants such as wheat, maize, potato, coffee, and banana. Colchicine, which induces polyploidization by inhibiting mitotic spindle formation, has been widely used to improve crops and horticultural species because tetraploids generated by colchicine treatment often produce organs larger than diploid parents (Levan 1938), thereby providing higher commercial values. Tetraploids of the model plant Arabidopsis thaliana are also larger than diploids, considering cell, organ, and body sizes (Tsukaya 2008).

Many plant species undergo somatic polyploidization, called endoreduplication (or endoreduplication), in which cells increase their DNA content without mitosis or cytokinesis after cell division cessation (Edgar et al. 2014; Sugimoto-Shirasu and Roberts 2003). Different plant species differ in endopolyploidy levels; for instance, abundant endopolyploidy is observed in the Brassicaceae family but is lower or undetectable in most woody plants (Bainard et al. 2012; Barow and Meister 2003; Nagl 1976). Until now, why woody plants do not undergo endoreduplication has remained unidentified because most studies on the molecular mechanisms underlying polyploidization have been conducted using plant species capable of inducing endoreduplication. Increasing the woody biomass supply is a pressing issue to reduce the cost of production of bioenergy and biomaterials (Ragauskas et al. 2006; Yang et al. 2006). However, only a few attempts have been made to induce polyploidization and to examine its effect on organ size in woody plants.

Here, we generated tetraploid poplars and conducted cell- and tissue-level analyses, focusing especially on the stem. As in plant species capable of inducing endoreduplication, tetraploidization increases cell and organ size in poplars, sacrificing longitudinal stem growth.
growth. Our findings indicate the usefulness of tetraploidization in woody biomass production and provide another strategy to achieve carbon neutrality.

Materials and methods

Plant materials and growth conditions

Hybrid aspen Populus tremula × Populus tremuloides clone T89 was subcultured every month by transferring the apical part of shoots to a 0.5 × 10^−3 Murashige and Skoog medium (pH 5.8) containing 0.3% (w/v) gellan gum. Plants were grown under 16 h light/8 h dark conditions at 22°C. For plant growth analysis, 4-week-old plants were transplanted into the soil and acclimated in a greenhouse for 10 days. Plants were then grown in a greenhouse at the Nara Institute of Science and Technology from May 11 to September 5, 2017. To collect samples for nuclear magnetic resonance (NMR) footprinting analysis, plants were grown from June 8 to October 19, 2015. Temperature was kept between 20°C and 22°C, and natural daylight was supplemented with the light from halogen lamps to maintain the 16 h light/8 h dark cycle.

Generation of tetraploid poplars

The apical part of shoots cultured in vitro for four weeks was removed to leave three leaves in the basal part, and a 0.01% (w/v) colchicine solution containing 0.2% (w/v) agar was dropped onto the leaf joints. After 24 h, the colchicine solution was removed, and the joint portion was washed with sterile water by pipetting. This colchicine treatment was conducted once more, and the plants were cultured for four weeks to grow lateral shoots from colchicine-treated lateral buds. Leaves were harvested from lateral shoots and subjected to DNA ploidy measurement using CyFlow space with FloMax flow cytometry (Partec). The lateral shoots were then cut off and transferred to a new medium for cultivation for four weeks. After subculturing two times more, lines that displayed only the 4C peak in ploidy analysis were isolated as tetraploids.

Microscopic observations

Free-hand stem sections of poplars cultured in vitro were made with a razor blade and briefly stained with 0.1% (w/v) toluidine blue. After washing with water, the sections were observed under bright field microscopy SZX16 (Carl Zeiss).

Plant growth analysis

To monitor plant growth in a greenhouse, the height and diameter of the stem and leaf number were measured every week. The stem diameter was measured at 10 cm above the ground. The leaf area was measured after 17-week cultivation. To estimate the fresh and dry weights of the stem, 17-week-old plants were cut off at the base, and the whole stem without leaves was weighed, followed by drying at 60°C for 10 days in an oven to estimate the dry weight.

Density measurement of wood substance

A five-centimeter stem was cut out from part 1 (0–15 cm from the ground), part 2 (16–61 cm from the ground), and part 3 (92–107 cm from the ground). Wood substance density was measured using a water replacement method (Aso 1951). First, water was impregnated under a vacuum condition for six days, and weight was measured in water to estimate buoyancy. Second, the specimen was air-dried at 60°C for a day and vacuum-dried at 60°C for another day. The dry weight of the 5-cm stem (Wd) was scaled as a vacuum-dried specimen, and wood substance volume (Vs) was estimated, assuming that the water density is 1.0 g cm⁻³. Wood substance density (ρs) was calculated as follows:

\[ \rho_s = \frac{W_d}{V_s} \]

Enzymatic saccharification analysis

Powdered shoot samples were treated with a mixture of cellulase from Trichoderma reesei ATCC 26921 (Sigma-Aldrich, Merck KGaA) and cellobiase from Aspergillus niger (Sigma-Aldrich, Merck KGaA) for 24 h following previous methods (Ohtani et al. 2017; Okubo-Kurihara et al. 2016; Nakano et al. 2022). After adding 0.1 M NaOH solution to stop the reaction, the supernatant was collected by centrifugation. Released glucose was measured with a Glucose CII-Test (FUJIFILM Wako Pure Chemical Industries, Ltd. Corporation).

NMR footprinting analysis

The method for NMR footprinting analysis was essentially described by Hori et al. (2020), Akiyoshi et al. (2021), and Nakano et al. (2022). Briefly, stem samples were freeze-dried and debarked and then ground in an automill (TK-AM7, Tokken) for 10 min. The samples were then further ground in a Pulverisette 5 ball mill (Fritsch) for 12 h. Next, 30 mg of powdered sample was dissolved in dimethyl sulfoxide (DMSO)-d₆, pyridine-d₅ (4:1) and heated at 50°C for 30 min, shaking at 1,400 rpm in a Thermomixer Comfort (Eppendorf). After centrifugation at 15,000 rpm for 5 min, the supernatant was transferred into NMR tubes for analysis. The NMR spectra were collected on an Avance II HD-700 instrument (Bruker) with a 5-mm cryoTCI probe. The echo/antiecho gradient selections were used to collect 2D ¹H-¹³C hetero-nuclear single quantum coherence (HSQC) spectra. Fifty-one regions of interest (ROI) were defined based on previously assigned chemical shifts (Komatsu and Kikuchi 2013; Mori et al. 2015; Watanabe et al. 2014). HSQC quantification was performed following previously described protocols (Mansfield et al. 2012; Tsuji et al. 2015). Principal component analysis (PCA) was performed in R, and the lignin syringyl/guaiacyl/p-hydroxyphenyl units were calculated based on the intensity of the peaks corresponding to each lignin monomer structure.
Results and discussion

Establishment of tetraploid lines

As in other woody plants, poplar trees do not undergo endoreplication, except for seed hair cells (Kondorosi et al. 2000; Ye et al. 2014). To examine the phenotypic outcome of polyploidization in woody plants, we generated stable tetraploid lines of hybrid aspen (Populus tremula L. × Populus tremuloides Michx.) clone T89. Colchicine has been widely used to isolate polyploids from regenerating shoots in an in vitro tissue culture system, but this approach consumes much time and effort. We therefore developed an in planta system to induce polyploidization in poplars. The upper part of diploid plants grown in an agar medium was cut off, and 0.01% (w/v) colchicine was dropped onto the joint of the leaves remaining in the basal part. Four weeks later, leaves on lateral shoots derived from colchicine-treated lateral buds were subjected to DNA ploidy analysis: 54% (195/361) shoots displayed chimeric DNA levels of 2C, 4C, 8C, and 16C, whereas 26 tetraploids and 4 octaploids were also obtained (Supplementary Table S1). We then cut off lateral shoots and transferred them to a new medium, followed by 4-week cultivation. This subculturing process was repeated two times more, and we finally isolated 10 lines that gave only the 4C and extremely low 8C peaks, the latter of which represented the 4C cells after DNA replication, in ploidy analysis (Figure 1A). Notably, in most lateral shoots, ploidy levels decreased during subculturing, probably due to contamination of cells with lower DNA levels. DNA ploidy of the 10 isolated lines also exceeded 4C in the first generation but converged on 4C in the third generation and remained constant afterward; therefore, we used them as established tetraploid lines.

Tetraploids have larger cells in the stem than diploids

To first investigate whether tetraploidization affects cell size, we observed stem tissue, the major biomass source of woody plants. Since all tetraploid lines exhibited almost the same phenotype, we used three representative lines for detailed analysis (#3, #13, and #16). Transverse stem sections of plants cultured in vitro showed no difference in cellular organization between diploids and tetraploids (Figure 1B, C). Cell number in each radial cell file of the secondary xylem was the same between diploids and tetraploid lines #13 and #16 (Figure 1F), whereas the size of xylem fiber cells in tetraploids significantly exceeded that in diploids (Figure 1D, E, G). To examine whether increased cell size is a general feature of tetraploids, we also measured the cell size of the pith, cortex, and epidermis. Our data showed that tetraploid cells were larger irrespective of cell type (Supplementary Figure S1), suggesting that poplars maintain the ability to enlarge cells upon polyploidization.

Radial stem growth is promoted in tetraploids

To investigate the growth traits of tetraploid poplars, we transplanted the plants that were grown in an agar medium for four weeks, and nuclei were isolated from leaves. (B–E) Stem sections of diploids (2n) and tetraploids (4n). The 10th internode of plants cultured in vitro for 50 days was used for sectioning. Magnified images of the xylem tissue surrounded by white squares in (B) and (C) are shown in (D) and (E), respectively. Scale bars: 100 μm (B, C) and 25 μm (D, E). (F, G) Cell number and cell area in the xylem. The 10th internode of plants cultured in vitro for 50 days was sectioned and used for measurements. The cell number was counted in each radial cell file of the secondary xylem (F), and the cell area was measured for xylem fiber cells (G). Data are presented as mean±SD [n=150 for (F), and n=300 for (G)]. Significant differences from the diploid were determined using Student’s t-tests: ** p<0.01.
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getting larger as plants grew (Figure 2B). Slower growth of tetraploid poplars was also reported for another line generated by chromosome doubling of diploid hybrid progeny (Xu et al. 2020). However, to our surprise, the basal stem diameter was significantly larger in tetraploids when transferred to the greenhouse and afterward (Figure 2C). Consequently, radial growth per unit stem elongation was much faster in the tetraploid lines (Figure 2D), suggesting that radial growth was enhanced at the cost of reduced longitudinal growth. Due to slower stem elongation, the leaf number was decreased in tetraploids, but the size of younger leaves markedly exceeded that of diploids (Figure 2E, F). After 17-week cultivation, we measured fresh and dry weights of the whole stem and found that both showed no difference between diploids and tetraploids (Figure 2G, H). This and the above data suggest that faster radial growth compensated for slower stem elongation in tetraploids, providing an equal stem weight to diploids.

Qualitative assessment of woody biomass

To investigate cell wall properties, we collected...
stem samples from different positions after 17-week cultivation in a greenhouse: part 1 (0–15 cm from the ground), part 2 (46–61 cm from the ground), and part 3 (92–107 cm from the ground). The dry weight per 5-cm section of each part was almost proportional to the stem diameter, irrespective of the DNA ploidy levels (Figure 3A). We then calculated wood substance density, which represents the dry weight divided by cell wall volume. Although the data variability was greater in part 3, wood substance density did not significantly differ between diploids and tetraploids (Figure 3B).

To further estimate the cell wall properties, we measured enzymatic saccharification efficiency (Nakano et al. 2022; Ohtani et al. 2017; Okubo-Kurihara et al. 2016; Yang et al. 2006). Cellulose and hemicellulose in powder samples of whole shoots, which were grown in an agar medium for four weeks, were saccharified to quantify the amount of released glucose after 0-, 5-, or 24-h enzymatic incubation. The released glucose level was comparable between diploids and tetraploid lines #13 and #16, whereas line #3 displayed a slightly higher saccharification efficiency (Figure 3C). Next, we collected 20-cm stem sections from the basal part after 19-week cultivation in a greenhouse and performed NMR footprinting analysis to obtain further information on cell wall profiling (Chylla et al. 2013; Komatsu and Kikuchi 2013; Mansfield et al. 2012; Ragauskas et al. 2006). PCA based on 51 peaks derived from lignin and polysaccharide-related chemical groups revealed that none of the diploid or tetraploid plots were clearly clustered; rather, we observed a weak tendency that individuals from the same line were plotted relatively closely (Figure 3D). The NMR data also indicated that the ratio of three major lignin units—syringyl, guaiacyl, and \( p \)-hydroxyphenyl—was comparable between the diploid and tetraploid samples (Figure 3E). Overall, we identified no significant difference in woody biomass characteristics between diploids and tetraploids.

**Conclusion**

In this study, we generated tetraploid poplars from colchicine-treated lateral buds. We also succeeded in producing tetraploids of *Eucalyptus* in the same way (our unpublished result), indicating that this simple method without tissue culture can generally be applied to woody plant tetraploidization. Stem cells in tetraploids exceeded those in diploids, implying that polyploidization in trees causes cell enlargement, as observed in plant species undergoing endoreplication. The most surprising finding was that, in tetraploids, radial stem growth was greatly promoted at the expense of longitudinal growth without detectable changes in woody biomass characters, suggesting that tetraploids use the energy and assimilation products preferentially.
to radial growth. Therefore, synergizing technologies that enhance energy production and distribution, such as an improvement of photosynthetic activity in source organs or accelerating metabolite transport in the plant body, and tetraploidization could be a useful and efficient strategy to increase biomass accumulation in the stem, thereby contributing to solving global environmental problems. In future studies, field tests will provide useful insights into the physical properties of tetraploid trees.

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Author contribution

T.D., I.K., K.M. and M.U. designed the research. C.U.-H., H.I., M.O. and T.M. performed the experiments and analyzed data. C.U.-H., H.I., M.O., K.M. and M.U. wrote the article.

Conflict of interest

We have no conflict of interest.

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