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Note

Induction of tetraploid hardy rubber tree, *Eucommia ulmoides*, and phenotypic differences from diploid

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Abstract Polyploid plants tend to have larger organs than diploids due to the doubling of their chromosome number. However, the enlarged regions, whether cells, tissues, or individuals differ depending on the plant species or taxon, as have metabolic changes. *Eucommia ulmoides* is a deciduous dioecious plant natively distributed in China that accumulates *trans*-1,4-polyisoprene (TPI) in laticifers throughout its tissues. To induce tetraploids of *E. ulmoides*, colchicine treatment time and concentration were assessed for ability to obtain tetraploids, and the resulting plants were analyzed for phenotypic differences from diploids with respect to individual size, 16 leaf attributes, sizes of three cell types (epidermis, laticifer, and parenchyma), and TPI content and molecular weight. Effective conditions for obtaining tetraploids were 0.05% colchicine for 48 h, which led to obtaining a total of 9 tetraploids. The tetraploids showed larger stomata size but lower stomata density than diploids. Significant differences in the mean values of other attributes including leaf size, cell size, and TPI content of tetraploids could not be detected between tetraploids and diploids, suggesting that *E. ulmoides* is less affected by gene doubling than other plant species, and that tetraploid individuals might not have a tendency to increase their biomass.

Key words: Histochemical analysis, laticifer cell, leaf attributes, stomata, *trans*-1,4-polyisoprene.

Polyploidy is widespread in many plant taxon and plays an important role in the evolutionary process. In general, polyploid plants often possess larger cell sizes and sometimes larger biomass, height and girth (Comai 2005; Oselebe et al. 2006), leaf size and thickness (Stupar et al. 2007), fruit mass (Oselebe et al. 2006; Vandenholt et al. 1995), and stomata size (Miller et al. 2012; Mishra 1997; Speckmann et al. 1965). The phenotypic alterations sometimes affect secondary metabolites (Cohen et al. 2013; Dhawan and Lavania 1996). For example, polyploid plants in the Asteraceae contain less oil and lower oleic acid composition in fatty acids of seeds (Estilai 1993). The changes in both phenotype and metabolic activities are induced by duplicated gene actions. The duplicated genes relax constraints on some functions and lead to new phenotypes or increased tolerance to environmental change resulting in better selective adaptation to new environments (Osborn et al. 2003). However, entirely duplicated genes may not function because of gene silencing (Comai 2005). Because the increase in the number of chromosomes does not necessarily increase the size of all tissues or change expression of all genes, the relationship between changes in phenotype and ploidy vary depending on plant taxon.

Induction of polyploidy can often be utilized to create a new plant variety for agricultural use and industrial usage (Dhooghe et al. 2011). Cavendish bananas which are widely cultivated in the world, are a triploid derived from a wild banana, *Musa acuminate* (Jain and Priyadarshan 2009). Rapeseed (*Brassica napus*) is an allopolyploid plant in the mustard family and is considered to have spontaneously formed as a hybrid of *B. rapa* and *B. oleracea* about 7500 years ago (Chalhoub et al. 2014). The polyploids presumably occur in nature due to abnormal cell division including imperfect chromosome partitioning. To produce polyploids artificially, colchicine that is a toxic alkaloid originally found in the plant *Colchicum autumnal* (Colchicaceae) is

Abbreviations: BR, polybutadiene rubber; DBH, diameter at breast height; D_0, diameter at ground level; FCM, flow cytometry; FT-IR, Fourier transform infrared; GPC, gel permeation chromatography, $\overline{M_n}$, number-average molecular weight; MS, Murashige–Skoog; $\overline{M_w}$, weight-average molecular weight; SCLSM, spectral confocal laser scanning microscope; SEC, size exclusion chromatography; SLA, specific leaf area; SUMP, Suzuki's Universal Micro-Printing; THF, tetrahydrofuran; TPI, *trans*-1,4-polyisoprene.

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used. It inhibits chromosome partitioning by preventing microtubule polymerization and results in increasing the chromosome number of surviving cells. Since the degree of inhibition of colchicine on chromosome partitioning and mitosis varies depending on plant species due to differing sensitivity, colchicine concentration and exposure time, the parts of a plant treated and other factors should be examined in order to produce a new polyploid plant effectively (Harbard et al. 2012)

Our target plant species is *Eucommia ulmoides* Oliver which is a dioecious diploid ($2n=34$ and $1C=0.74$ pg; 725 Mbp) plant, and the only member of the family Eucommiaceae. It is an endangered plant species natively distributed in China. This plant accumulates *trans*-1,4-polyisoprene (TPI) throughout the entire plant (roots, bark, leaves, and fruit) and the leaves contain about 3–7% TPI by dry weight (Takeno et al. 2008). TPI particles fill the laticifer cells in *E. ulmoides* tissue (Nakazawa et al. 2013). TPI has elastic characteristics and has been utilized as an industrial raw material for submarine cables, golf balls and other end uses, and the leaves and bark of *E. ulmoides* have respectively been utilized for tea and Chinese medicine. For further production of TPI, breeding by polyploid plant induction seemed to offer the possibility of increasing the entire body mass, or the size of leaves or laticifer cells, which could lead to the mass production of TPI using *E. ulmoides*. In this study, we (1) evaluate the effects of colchicine on polyploid induction of *E. ulmoides*, (2) assess any differences in individual size, leaf attribute, and TPI content between diploid and tetraploid plants, and (3) evaluate the possibilities of industrial utilization of *E. ulomoides* with different levels of ploidy.

To induce the polyploids artificially, *E. ulmoides* seeds were dipped in 3% sodium hypochlorite solution for 20 min, washed with sterile distilled water, and planted in petri dishes with filter paper containing 10 ml of colchicine solution. After colchicine treatment, each seed group was washed again with sterile distilled water and planted on solid Murashige–Skoog (MS) medium. The seeds were incubated at 25°C under conditions of 16 h light at a photosynthetic photon flux density of 50 μmol m^{-2} s, and germinated seeds were transplanted to individual glass tubes with 10 ml solid MS medium. As a control, 50 seeds were treated with water, and germinated in the same way. To screen tetraploids, treated 40-day-old seedlings in glass tubes were analyzed individually by flow cytometry (FCM). 0.7 cm^2 of leaves per individual were harvested and shredded in extraction buffer (High Resolution DNA Kit A, Partec, Görlitz, Germany) using a razor, and then the buffer containing extracted nuclei was filtered through a 30 μm filter (CellTrics, Partec). As a staining buffer, the extraction buffer was prepared by adding DAPI and four times amount of the staining buffer was added to each sample solution, incubated for 5 min at room temperature and loaded to the FCM (Ploidy Analyzer, Partec). As an internal reference standard, known diploid samples were mixed with the unknown samples described above.

Generally, the FCM histograms show that the diploids have only one peak, which have relatively low fluorescence intensity due to the presence of two sets of chromosomes, while on the other hand, the tetraploids have one peak of higher fluorescence intensity than the diploid set due to the presence of four sets of chromosomes. Of 600 *E. ulmoides* seeds treated under various conditions in 2004, 173 seedlings were obtained, and 29 of them showed the high fluorescence intensity peak, suggesting that they were tetraploids (Table 1, Figure 1). In addition, several individuals showed a histogram that had two peaks, one with high

Table 1. Effects of different concentration of colchicine and treatment time on occurrence of polyploid individuals. 100 seeds were used for each treatment and germinated on solid MS medium containing 20 g l^{-1} sucrose (Wako, Osaka, Japan) and 2.4 g l^{-1} Gelrite (Wako).

Colchicine concentration (%)	Treatment time (h)	No. surviving and analyzed individuals	No. tetraploids	
			Individuals	%
0.01	24	55	5	9.1
	48	48	4	8.3
	72	27	6	22.2
0.05	24	18	3	16.7
	48	18	9	50.0
	72	7	2	28.6

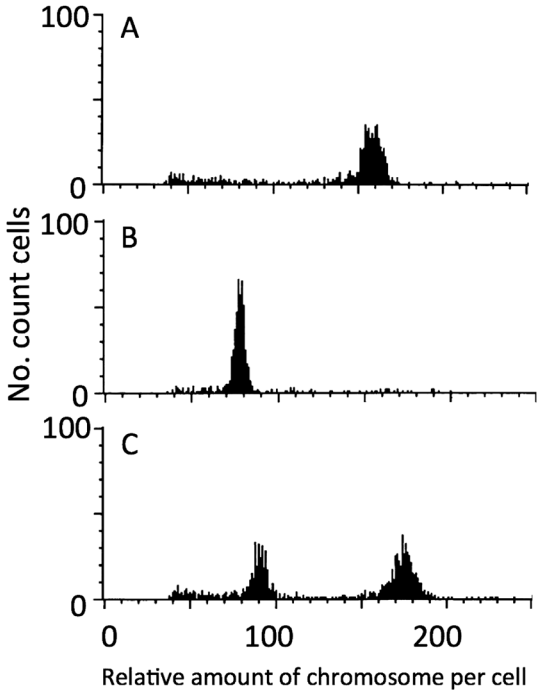

Figure 1. Histogram of relative chromosome content per cell based on FCM analysis. (A) Tetraploid, (B) Diploid, and (C) Chimera.

and one with low intensity, suggesting that they were chimeric (Figure 1). Following colchicine treatment, the number of surviving individuals decreased with an increase in concentration and exposure time; however, lower concentrations and shorter exposure times led to a decreased percentage of tetraploids (Table 1). The most stringent conditions for obtaining tetraploids were 0.05% colchicine for 48 h; half of these individuals were tetraploid (Table 1). When seeds were treated in 0.01% colchicine for 24 h, about half of the seeds germinated, but only five individuals were tetraploid.

Colchicine causes serious cellular toxicity to plants, so higher concentrations and longer treatment times could lead to a lower survival rate, despite effective induction of polyploids (Zeng et al. 2006). For *Acacia crassicarpa* (Fabaceae), the effective concentration was 0.001–0.020% (Harbard et al. 2012). For plants in the genera of *Paulownia* (Scrophulariaceae) and *Platanus* (Platanaceae), it was 0.05% and 0.4%, respectively (Liu et al. 2007; Tang et al. 2010). The score of treatment conditions for *E. ulmoides* was within the range of these woody plants; its sensitivity to colchicine might lie about halfway within the scores of treatment conditions reported for other woody plants.

The obtained 29 tetraploid individuals were planted and cultivated in the testing field of the Faculty of Agriculture, Kyushu University (33°37′N, 130°25′E, Fukuoka, Japan). After 10 years from colchicine treatment (November 2014), the FCM analysis of survived individuals in the testing field was performed and confirmed their ploidies again. Eleven individuals were growing and of them, three individuals showed

Table 2. Summary of the measured attributes with average (±S.D.) values for tetraploid and diploid.

Plant attribute	Ploidy		χ^2	df	p*
	Tetraploid	Diploid			
D_0 (mm)	18.9±12.2	31.3±19.9	1.64	1	0.20
DBH (mm)	18.1±4.33	20.1±13.7	0.18	1	0.67
Plant height (cm)	136±93.0	250±81.6	2.82	1	0.09
Stomata length (mm)					
Major axis	37.6±5.67	31.2±7.22	8.21	1	**<0.01**
Minor axis	7.51±1.95	5.38±1.78	17.81	1	**<0.01**
Stomata density (/μm^2)	1.13±0.31	1.72±0.49	10.49	1	**<0.01**
Leaf dry weight (mg)	0.52±0.34	0.39±0.30	0.05	1	0.82
Leaf length (cm)	13.2±4.43	13.2±3.47	0.22	1	0.64
Base blade length (cm)	4.70±1.90	4.70±1.30	0.10	1	0.74
Leaf width (cm)	7.30±2.19	6.25±1.38	0.45	1	0.50
Petiole length (cm)	1.54±0.50	1.71±0.38	0.66	1	0.42
Leaf area (cm^2)	73.9±37.8	57.5±27.5	0.22	1	0.64
Perimeter (cm)	50.2±18.2	41.7±10.8	0.50	1	0.48
Circularity	0.37±0.09	0.40±0.08	0.87	1	0.35
SLA (cm^2/mg)	169±47.6	213±92.4	0.27	1	0.61
Petiole height (μm)	1.95.E+03±607	1.63.E+03±714	0.31	1	0.58
Petiole width (μm)	2.39.E+03±610	1.56.E+03±624	3.49	1	0.06
Vascular bundle height (μm)	420±127	308±124	1.58	1	0.21
Vascular bundle width (μm)	1.25.E+03±270	880±296	3.08	1	0.08
Epidermal cell height (μm)	17.3±10.2	13.5±3.06	1.14	1	0.29
Epidermal cell width (μm)	14.9±9.94	10.5±2.51	1.13	1	0.29
Laticifer diameter (μm)	8.76±2.51	8.42±2.63	0.10	1	0.76
Parenchyma diameter (μm)	57.3±18.7	46.2±10.9	1.23	1	0.27
TPI Content per leaf (%)	1.96±0.74	1.54±0.53	1.15	1	0.28
Total amount of TPI per leaf (mg)	15.4±8.54	9.50±8.14	0.45	1	0.50
Molecular weight of long-chain TPI					
Mn	1.38.E+06±2.60.E+05	1.06.E+06±3.42.E+05	2.66	1	0.10
Mw	3.63.E+06±7.07.E+05	2.87.E+06±1.08.E+06	1.72	1	0.19
Mw/Mn	2.61±0.19	2.71±0.20	1.35	1	0.24
Molecular weight of short-chain TPI					
Mn	7.76.E+03±4.74.E+02	7.86.E+03±1.08.E+03	0.02	1	0.89
Mw	1.82.E+04±2.28.E+03	1.79.E+04±4.28.E+03	0.05	1	0.82
Mw/Mn	2.31±0.17	2.27±0.25	0.17	1	0.68

*Boldface type indicates significant differences between tetraploid and diploid. To assess the differences in individual sizes, generalized linear modeling with Gaussian distributions and identity link functions was conducted. D_0, DBH, and tree height attributes were included as explanatory variables, and the ploidy of each individual was set as responce variable. The differences in leaf attribute and TPI characteristics were used to run generalized linear mixed models with Gaussian distributions and identity link functions (Bolker et al. 2009). Each attribute index was set as an explanatory variable, the ploidy of each individual was set as a fixed effect and individual plants and branches nested in the individual plant were set as random effects. All statistical analyses were performed by R ver. 3.1.2 software (R Core Team, Vienna, Austria) with the car and lme4 packages.

diploid and eight were tetraploid (Figure 1). The diameter at ground level (D_0), diameter at breast height (DBH), and height of individual plants were measured and the mean of the individual tetraploid plants were 18.92 mm, 18.09 mm, and 135.56 cm, respectively (Table 2). The diploid individuals showed almost the same values for all size variables ($p>0.05$). These individuals were cultivated in the testing field under almost the same conditions; no significant growth differences between tetraploids and diploids were observed.

Following their measurement of D_0, DBH, and height, three branches per individual plant were selected and three leaves per branch were harvested. To analyze stomata form, the abaxial surface of the leaf was copied on film by Suzuki's Universal Micro-Printing (SUMP) methods using SUMP liquid and the film was observed under a microscope; on each film, major and minor lengths of nine stomata were measured and the number of stomata per 0.01 mm^2 (100 μm×1000 μm) was counted. Both major and minor lengths of stomata of tetraploids were about 1.2 times bigger than that of diploids ($p<0.01$). In contrast to stomata size, the stomata density of diploids was significantly higher than that of tetraploids ($p<0.01$, Table 2).

Generally, polyploid cells possess doubled chromosomes in a single nucleus, which causes a moderate increase in the cell size (c. 1.6-fold increase; Comai 2005). In this context, polyploid stomata-associated guard cells are often larger than those of diploids in many plant taxon, and these structures have been utilized as an indicator of polyploidy (Miller et al. 2012; Speckmann et al. 1965). Like other polyploid plants, *E. ulmoides* tetraploids showed larger guard cells and stomata due to doubled chromosome number, so these can be utilized as a marker to identify tetraploid individuals in *E. ulmoides*. In contrast, the stomata density of *E. ulmoides* tetraploid leaves was lower than diploids (Table 2). Polyploid plants often have negative relationships between stomata size and stomata density (Khazaei et al. 2010; Miller et al. 2012), and the regulation mechanisms between stomata size and stomata density are not known yet. Recent study indicated that stomata density was controlled by stomagen in *Arabidopsis* (Lee et al. 2015). Increased numbers of chromosomes in polyploids often changes a lot of gene expressions, resulting in phenotypic alteration including stomata density in plant (Comai 2005). The regulation mechanisms of stomata density on the molecular level is still unrevealed, decreased stomata density might be controlled by stomagen-like peptide in *E. ulmoides*.

To analyze the shapes of diploid and tetraploid leaves, a maximum of 10 leaves from each branch were harvested and scanned, and seven leaf attributes (leaf length, width, base blade length, petiole length, leaf area, perimeter, and circularity) were measured by ImageJ software (Rasband, W.S., Bethesda, MD) (Schneider et al. 2012) (Table 2). Although the mean values of leaf attributes of tetraploids, except for petiole length, seemed bigger than those of diploids, no significant differences were observed ($p>0.05$, Table 2).

After using the leaves for shape analysis, they were divided into three groups; one was for frozen section observation using microscopy, another for analysis of TPI content and determination of dry weight of leaf, and the third group was for determination of dry weight of leaf. The leaves for TPI analysis and for dry weight calculation were dried at 60°C for 48 h in a vacuum oven and weighed to the nearest 0.01 mg. The dry weights of leaves and the leaf areas were used to calculate the index of leaf weight per unit area (specific leaf area or SLA) for the leaves of the second and third groups.

For microscopic observation, petioles of the leaves were dipped in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), snap-frozen in liquid nitrogen, and sliced into thin sections of 12 μm thickness with a stainless steel blade at −25°C using a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany). The height and width of petioles, vascular bundle, and epidermis were measured to the nearest 0.1 μm (Table 2). The sections were stained with 30 $\mu g\,ml^{-1}$ Nile red and 1 $mg\,ml^{-1}$ Fluorescent Brightener 28 dissolved in EtOH. The cell wall structure and laticifers with TPI were analyzed by a spectral confocal laser scanning microscope (SCLSM) system (Nikon TE2000 E, Digital Eclipse C1si, EZ-C1 3.40 software; Nikon, Tokyo, Japan) equipped with two lasers (405 and 488 nm). The emission spectra of TPI and lipophilic chemicals were detected from 500 to 650 nm with a 5 nm bandwidth and 32 channels, and the emission spectrum of the cell wall was detected from 460 to 470 nm with a 5 nm bandwidth and 3 channels. Four scanned images were superimposed and spectral unmixing was performed using the reference spectra of TPI and lipophilic chemicals (Nakazawa et al. 2013). For the details of the SCLSM analysis methods, see Nakazawa et al. (2013). Diameters of three laticifers and three parenchyma cells per leaf were measured to the nearest 0.01 μm twice and the scores averaged (Figure 2, Table 2).

The mean value of tetraploid SLA, an index of leaf thickness, seemed less than that of diploids; however, the scores did not differ between tetraploids and diploids. The cell size, petiole width and vascular bundle width of tetraploids seemed 1.2 times bigger than those of diploids, but there were no significant differences ($p>0.05$, Table 2, Figure 2). Laticifer and parenchyma diameters and epidermis size were almost the same size for tetraploids and diploids (Table 2, Figure 2). For many plant polyploids, scores for individual leaf areas or leaf thicknesses are higher than those of the normal diploid

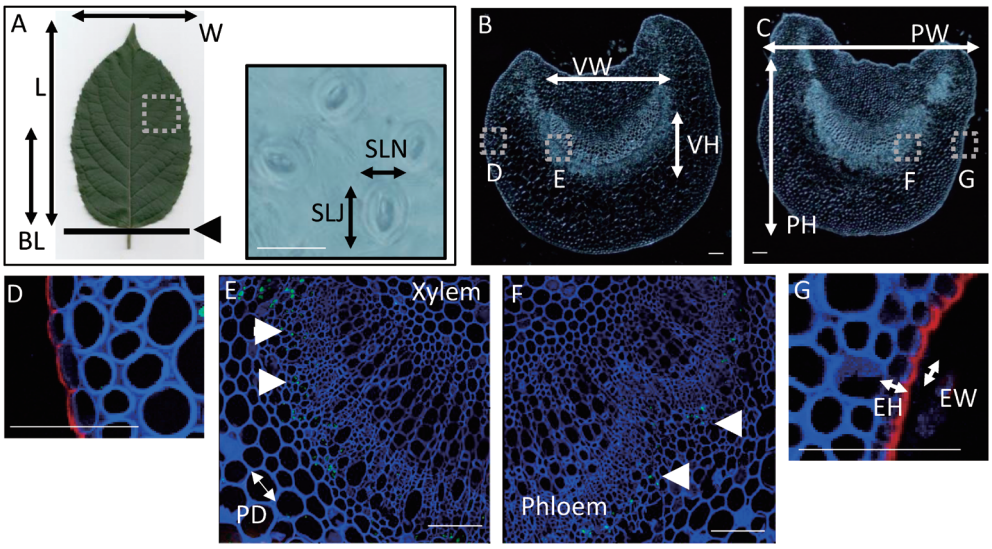

Figure 2. Images of the stomata, leaf, petiole, epidermis, and vascular bundle with measured attributes of tetraploids and diploids. (A) Left: diagrams showing leaf attributes: L, leaf length; BL, base blade length; W, leaf width. The line and black triangle indicate the slice position for the frozen petiole section. Right: magnified image of white dotted square in left image and the stomata on the abaxial side of a leaf captured by SUMP film. Each abbreviation means the measurement site: SLJ, stomata major length; SLN, stomata minor length; bar indicates 10 mm. (B) light-field image of petiole cross-section of tetraploid. (C) Petiole of diploid. (D) Unmixed image of epidermal cell of tetraploid. (E) Vascular bundle of tetraploid, (F) Epidermal cell of diploid, and (G) Vascular bundle of diploid. (B) and (C) are light-field images; bars indicate 1000 μm. Each abbreviation means the measurement sites: PH, petiole height; PW, petiole width; VH, vascular bundle height; VW, vascular bundle width. (D), (E), (F), and (G) are unmixed images measured by SCLSM; each bar is 100 μm. Display color: TPI, green; lipid-soluble, red; cell wall, blue. White triangles show the site of laticifer cells. Each abbreviation means the measurement site: PD, parenchyma diameter; EH, epidermal cell height; EW, epidermal cell width.

plants due to a larger increment of cell size (Miller et al. 2012; Oselebe et al. 2006; Stupar et al. 2007), but the tendency toward increased cell size in *E. ulmoides* was less than that of other plant species.

The TPI content and molecular weight were analyzed using a Fourier transform infrared (FT-IR) spectrophotometer (IRAffinity-1; Shimadzu Corp., Kyoto, Japan) and size exclusion chromatography (SEC), respectively, following the method described by Takeno et al. (2008). The second group leaves were ground into powder in liquid nitrogen using a mortar and pestle and the samples were additionally divided into two groups: one containing samples weighing about 1.5 mg for analysis of TPI content, which were placed in 2 ml tubes with three replications per leaf, and the other contained samples weighing 150 mg for analysis of TPI molecular weight, which were placed in 15 ml tubes without replication. For TPI content analysis, 1 ml EtOH was added to the tubes, and for molecular weight analysis, 10 ml EtOH was added; the tubes were heated at 60°C for 5 min and centrifuged (10,000 rpm, 1 min), and the supernatant was removed. The treatment was repeated until the supernatant was colorless (c. 5–6 times). After decolorization, toluene with the same amount of EtOH was added to the tubes, and the tubes were heated at 60°C for 30 min in a water bath. The toluene extract was decanted to a new glass tube and dried by an evaporator at 60°C.

A 1-ml aliquot of toluene solution with polybutadiene rubber (BR) dissolved at a concentration of 1 mg ml^{-1} was added to the TPI samples described above as an internal standard, and the TPI was dissolved completely. The solutions were dried in the vacuum dryer, dissolved again in 100 μl of chloroform, and a 10 μl aliquot of the chloroform solution was placed on a NaCl cell. A film containing the TPI and BR was obtained by evaporation of chloroform from the NaCl cell. Since the absorption peak of BR was at 912 cm^{-1} and TPI was at 1380 cm^{-1}, the amounts of TPI and BR were calculated using the peak areas of BR and TPI for the corresponding wave number by FT-IR. TPI content of each sample was calculated from the ratio of BR and TPI using a calibration curve between BR and predetermined amounts of TPI (31.25, 62.5, 125, 250, 500, 1000 μg ml^{-1} in toluene solution) on each day of measurement. TPI content as a percentage of each sample was calculated from the sample dry weight and TPI content. The average TPI content of each leaf was calculated from the three samples in each leaf. The total amount of TPI in a single leaf was calculated using the average TPI percentage and dry weight of whole leaves. The TPI content per leaf of tetraploids was 1.96±0.75% (mean±S.D.) and that for diploids was 1.54±0.53%, with no significant differences detected between tetraploids and diploids (p=0.28, Table 2). The total amount of TPI in a single leaf also did not differ significantly between tetraploids and diploids (p=0.50).

To analyze the molecular weight of extracted TPIs, SEC analysis was performed using a Tosoh Eco-SEC

HLC-8320GPC chromatograph (Tosoh, Tokyo, Japan). The extracted TPIs described above were dissolved in 2 ml tetrahydrofuran (THF) and filtered through a 0.45 μm membrane. A guard column (TSKgel guardcolumn Hhr (S) (7.5 mm I.D.×7.5 cm)) and two TSKgel GMHhr-H (S) (7.8 mm I.D.×30 cm) columns having a molecular weight exclusion limit of 400,000,000 were employed. THF was used as an eluent, with a flow rate of 0.5 ml min^{-1} at 40°C, and TPI was monitored by a refractive index detector. Polystyrene standards dissolved in THF were used for calibration. Because the natural TPI in *Eucommia* leaves has two peaks around 6000 and 1,200,000 molecular weight, number-average molecular weight (M_n), weight-average molecular weight (M_w), and the ratio of M_n and M_w (M_w / M_n) of each peak were calculated. The M_n of long-chain TPI was about 1,000,000 and the M_n of short-chain TPI was about 7,000. No differences between tetraploids and diploids in the molecular weight of either long or short chains were detected (p=0.10, p=0.89, respectively, Table 2). Other indices, M_w and M_w / M_n, also did not differ between tetraploids and diploids (Table 2).

A unique characteristic of *E. ulmoides* is its ability to synthesize TPI in its tissues and TPI is synthesized in laticiferous cells. Since in contrast with the enlargement of guard cells, no corresponding changes in laticiferous cells were observed, TPI production in leaves of each individual should be unchanged. Another plant that produces polyisoprene, guayule, which produces a latex containing *cis*-polyisoprene as well as resins and triglycerides in its seed oil, is known to generate polyploids spontaneously, and its tetraploids contain more seed oil than diploids (Estilai 1993). However, the rubber content and molecular weight vary by growing site, and differences dependent on ploidy were relatively small (Rodríguez et al. 1993). Although studies assessing the effects of different nutrient conditions on the polyisoprene content of *E. ulmoides* are unknown, and different numbers of chromosomes sometimes cause different metabolic activity (Comai 2005), secondary metabolites, such as rubber and other polyisoprenes, might not be as affected by the number of chromosomes as by nutrients in the soil.

In conclusion, appropriate conditions to induce and obtain *E. ulmoides* tetraploid individuals were established in this study. Although the tetraploid individuals did not show any distinctive characteristics except for stomata size and density, they were stably grown for 10 years. TPI of *E. ulmoides* is expected to be utilized as a raw material for isoprene that is not of fossil fuel origin. The tetraploids created in this study have not yet produced flowers. Polyploids frequently show phenotypic differences, like larger fruit, flower, and leaf sizes, improved stress tolerance (Dhooghe et al. 2011). Continuous studies of *E. ulmoides* including these reproductive organs might lead to further knowledge of polyploidy and the production of TPI.

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