Plant Cell, Tissue and Organ Culture (PCTOC) (2021) 146:285–296
https://doi.org/10.1007/s11240-021-02068-5

ORIGINAL ARTICLE

In vitro induction of tetraploid and resulting trait variation in *Populus alba* × *Populus glandulosa* clone 84 K

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Received: 17 December 2020 / Accepted: 20 March 2021 / Published online: 29 April 2021

Abstract

As a model plant, poplar 84 K (*Populus alba* × *P. glandulosa*) plays a key role in fundamental research in forest molecular biology. Poplar 84 K is a suitable plant for in vitro polyploidy induction and to study the consequent trait variation among perennial trees. In this study, the results indicated that the concentration of NAA (naphthaleneacetic acid) and BA (benzyladenine) has substantial effects on the differentiation rate of adventitious shoots, and media consisting of 0.5 mg L^{-1} BA and 0.05 mg L^{-1} NAA yielded the greatest number of shoots per explant. A total of 98 tetraploids were successfully obtained by colchicine treatment on diploid leaves in vitro. The rate of tetraploids was substantially affected by pre-culture duration, colchicine concentration and exposure duration. The highest tetraploid induction efficiency was 37.03%, which was achieved by treating leaves with 50 mg L^{-1} colchicine for 3 days after 4 days of pre-culture. With increasing ploidy, obvious morphological differences were discovered between tetraploids and diploids. Compared with the diploids, the tetraploids had larger and thicker leaves, larger but sparser stomata, fewer and shorter roots, and larger protoplasts. These tetraploids serve to enrich the polyploid germplasm resources of *Populus*. Moreover, our study lays an important groundwork for polyploid gene function and is important for exploring trait variation among *Populus* trees with different ploidy levels.

Key message

In vitro induction of tetraploid by colchicine treatment and differences in morphological features betweentetraploid and diploid plants.

Keywords Tetraploid · Colchicine · Chromosome doubling · Morphological features · Trait variation · *Populus*

Communicated by Henryk Flachowsky.

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Abbreviations

MS	Murashige and Skoog (1962)
BA	Benzyladenine
NAA	1-Naphthaleneacetic acid
IBA	Indole butyric acid
DAPI	4,6-Diamidino-2-phenylindole
MOPS	4-Morpholinepropane sulfonate
CPW	Cell protoplast washing medium
MES	4-Morpholine ethane sulfonic acid
BS	Bovine serum albumin
LSD	Least significant difference
SE	Standard error
SEM	Scanning electron microscopy

Introduction

Polyploidization has long been recognized as a prominent force in the evolution of plant species and is an important contributor to speciation (Stebbins 1971; Comai 2005; Wood et al. 2009). Polyploid plants generally present rapid growth, large vegetative organs and high secondary metabolite contents, and polyploidy is being used as an important strategy in tree genetic improvement (Zhu et al. 1995, 1998; Sattler et al. 2016; Liao et al. 2016; Parsons et al. 2019; Guo et al. 2019; Kang 2020). There are many ways to obtain polyploidy trees (Nilsson-Ehle 1936; Johnsson 1945; Weisgerber 1980; Einspahr 1984). Among them, somatic chromosome doubling is one of the quickest methods to obtain polyploidy (Mattila 1961; Thao et al. 2003; Zhang et al. 2008; Nilanthi et al. 2009; Cai and Kang 2011). Polyploidy can also be induced through the application of antimitotic agents to seeds, seedlings, in vivo shoot tips, or in vitro explants (Dermen 1940; Petersen et al. 2003; Talebi et al. 2017; Parsons et al. 2019). Among them, a controlled environment can be provided by inducing polyploids in vitro, and the test results are repeatable, greatly improve the efficiency and effects of somatic chromosome doubling. In recent years, with the development of plant tissue culture technology, inducing polyploids in vitro has been applied in more and more plants. At present, polyploid forest trees have been successfully obtained in vitro, including those of black locust (Ewald et al. 2009), *Paulownia australis* (Wang et al. 2017), *Populus* (Xu et al. 2016, 2018; Liu et al. 2018; Zeng et al.2019), sweetgum (Zhang et al. 2017), *Ziziphus* (Gu et al. 2005; Cui et al. 2017), mulberry (Wang et al. 2011) and *Betula* (Särkilahti and Valanne 1990).

Poplar 84 K (*Populus alba* × *P. glandulosa*) is a well-known hybrid. This hybrid resulted from a breeding programme led by Professor Sin Kyu Hyun (Seoul National University, Korea) and was first introduced into China in 1984 by Professor Qiwen Zhang (The Research Institute of Forestry, Chinese Academy of Forestry, Beijing) (Wang et al. 2005; Qiu et al. 2019). Poplar 84 K is currently popular in China because of its fast growth, high wood quality, strong resistance and broad adaptability. Importantly, poplar 84 K has been widely used by scientists in transgenic experiments as a model of woody species due to its high rate of transformation and differentiation (Shim et al. 2013; Yoon et al. 2014; Zhao et al. 2018; Zhang et al. 2019; Shu et al. 2019). Qiu et al. (2019) reported the whole genome and identified two subgenomes via comparison, opening up broader areas in forest molecular biology research by using 84 K poplar as an important material. At this time, tetraploids of poplar 84 K can be obtained by somatic chromosome doubling. This study not only provides polyploid germplasm resources for *Populus alba* × *P. glandulosa* but also plays an important role by exploring the molecular mechanism of forest polyploid trait variation.

In this study, we used diploid 84 K poplars as research objects. We then optimized the differentiation system of poplar 84 K leaves by applying gradients of different concentrations of hormones. On this basis, we have essentially conducted new research on polyploid induction systems via colchicine treatment on diploid poplar 84 K leaves in vitro. In addition, we observed and studied the growth status, leaf morphology and stomatal characteristics of both diploids and tetraploids.

Materials and methods

Plant materials

Poplar 84 K (*Populus alba* × *P. glandulosa*) were provided by the Beijing Forestry University greenhouse staff. All the plants were cultivated in a greenhouse whose light, temperature (10–20 °C), and humidity were controlled. The young stems of poplar 84 K, which served as explants, were washed with running water for 30 min. They were subsequently sterilized in a solution of 70% (v/v) ethanol for 30 s and 1% (v/v) sodium hypochlorite for 10 min and then rinsed in sterile distilled water three times. Finally, thoroughly cleaned explants with at least one bud were inoculated onto solid MS media (Murashige and Skoog 1962). After approximately 20 days, new adventitious shoots were transplanted to ½-strength MS media consisting of 0.05 mg L^{-1} IBA and 0.02 mg L^{-1} NAA for root formation. The pH of all the media was adjusted to 5.8–6.2, after which the media were autoclaved at 121 °C for 15 min. Rooting media was incubated at 25 °C under white fluorescent lighting (16 h photoperiod, average light intensity of 30–40 μmol m^{-2} s^{-1}).

Adventitious shoot regeneration from poplar 84 K leaves

After approximately 30 days, fully expanded leaves were harvested from sterile rooted plantlets and wounded with two transverse cuts crossing the main vein without full separation. The explants were placed on the differentiation media [MS media consisting of 0.5% (w/v) agar, 3% (w/v) sucrose, and different concentrations of NAA and BA, as shown in Table 1], with the adaxial side touching the media, to evaluate the effects of plant growth regulators on shoot formation of poplar 84 K. The number of adventitious shoots per explant was recorded after 30 days of culture, and the percentage of regenerated explants was calculated. The

Table 1 Effects of BA and NAA concentrations on shoot regeneration from leaf explants of poplar 84 K

Treatment	BA (mg L^{-1})	NAA (mg L^{-1})	Shoot formation rate (%)	No. of shoot per explant
1	0.30	0.03	46.67 ± 4.71	7.32 ± 0.72
2	0.30	0.05	63.33 ± 4.71	7.76 ± 0.50
3	0.30	0.07	80.00 ± 0.00	8.13 ± 0.56
4	0.50	0.03	70.00 ± 8.16	8.19 ± 0.14
5	0.50	0.05	96.67 ± 4.71	10.15 ± 0.46
6	0.50	0.07	83.33 ± 4.71	8.66 ± 0.005
7	0.70	0.03	73.33 ± 4.71	8.50 ± 0.23
8	0.70	0.05	83.33 ± 4.71	8.68 ± 0.18
9	0.70	0.07	93.33 ± 4.71	8.96 ± 0.065

The data represent the mean ± SD of three replicates

experiments were repeated three times, with ten explants per treatment.

Colchicine treatment for inducing polyploidy and plant recovery

Fully expanded leaves were cut twice without separation and cultured on solid adventitious shoot regeneration media [MS media consisting of 3% (w/v) sucrose, 0.2% (w/v) agar, 0.5 mg L^{-1} BA and 0.05 mg L^{-1} NAA] for 3, 4, or 5 days. Afterwards, the cultures were transferred to the same liquid media containing filter-sterilized colchicine at concentrations of 40, 50, or 60 mg L^{-1} for 2, 3, or 4 days of treatment. Each treatment consisted of 10 replicates and was repeated three times. After colchicine treatment, the explants were washed three times with sterile water and then transplanted into shoot regeneration media, after which they were allowed to grow for 4 weeks. The single adventitious shoot was then excised and placed on ½-strength MS media supplemented with 0.05 mg L^{-1} IBA and 0.02 mg L^{-1} NAA for root formation. Once the explants had recovered and produced at least three fully expanded leaves, survival rates were recorded, and each plant was sampled for flow cytometric ploidy analysis.

Flow cytometry is a rapid, reliable and simple method to measure the ploidy level and confirm the success of polyploidy induction, allowing the analysis of a large number of target plants in a short period of time (Galbraith et al. 1983; Roy et al. 2001). Young leaves were collected from healthy cultured plants. The leaf samples were chopped with a razor blade in a dish containing 1 mL of modified Galbraith's buffer [45 mM $MgCl_2 \cdot 6H_2O$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (v/v) Triton X-100 (pH 7.0); Doležel et al. 1989]. The suspension was then passed through a 30 µm nylon mesh filter to isolate the nuclei. The filtrate was subsequently stained with 80 µL of DAPI (5 mg mL^{-1}) for 5 s. The ploidy level was analysed by a Cyflow® Ploidy Analyzer (Partec PAS, Germany). The fluorescence intensity of the diploids was adjusted to 50, and tetraploids were identified as those whose peaks occurred at a relative fluorescence intensity of 100.

Chromosome number

Chromosome counting is considered the most accurate method to detect polyploid variants. The ploidy level of the regenerated tetraploid and diploid shoots was confirmed by chromosome counting. Young, unexpanded leaves were collected and pretreated with a saturated solution of paradichlorobenzene for 4 h, after which the leaves were then fixed in a 3:1 ethanol:acetic acid mixture for 24–48 h at 4 °C. After three rinses with water, the materials were dissociated in 1 N HCl for 20 min at room temperature. The material was subsequently squashed in modified phenol solution (Carbol fuchsin consisting of phenol solution and basic fuchsin) on a microscope slide. Then pressed down with a cover slip and tapped it a few times with pencil. The cells were imaged using an Olympus BX51 microscope and observed under a 100 × oil immersion objective lens.

Stomatal characteristics and phenotypic analysis

Twenty of the fully expanded poplar 84 K leaves of 10 tetraploids and 10 diploids were used to determine stomatal size and density. A few strips of the abaxial epidermis were added to a drop of water on a microscope slide and covered with a cover slip (Sari et al. 1999). Under an Olympus BX51 microscope, stomatal size was calculated on the basis of 100 randomly selected stomata, and thirty randomly selected microscopic fields were analysed to the measure stomatal density (Cui et al. 2017). To compare the differences between the tetraploid and diploid plants, the leaf size and the number and length of the roots of three selected individuals for each ploidy level were recorded.

Comparison of leaf mesophyll protoplast cell size

To analyse the size of mesophyll protoplast cells, fully expanded diploid and tetraploid leaves (1 g in total) were randomly selected and cut into approximately 1 mm thin strips. After pretreatment, the strips were transferred into an enzyme solution consisting of 3.0% (w/v) Cellulase Onozuka R-10, 0.05% (w/v) Pectinase Y-23, 0.2% (w/v) Macerozyme R-10, 109 g/L mannitol, 0.6 g L^{-1} MES, and 1 g L^{-1} BSA in CPW wash media at pH 5.8 (Huang 2015). The flask was sealed and then incubated under darkness and oscillation (20 rpm) at 27 °C for 7 h. After enzymolysis, the contents were filtered with a 200 mesh cell strainer and then transferred to a 10 mL centrifuge tube. The filtrate was centrifuged at 1000 rpm for 3 min, and the volume was fixed to 10 mL with CPW solution. Under an Olympus BX51 microscope, 100 protoplasts were randomly selected from diploids and tetraploids.

Data and statistical analysis

Univariate GLM was used to analyse the difference in effects between BA and NAA on the shoot formation rate and the number of adventitious shoots per explant. We also used the same method to analyse the differences in tetraploid induction rates among pre-culture duration, colchicine concentration, and exposure duration. For the experimental data measured as a percentage, arcsine transformation was used before one-way analysis of variance. When treatments differed significantly, LSD multiple comparison tests were performed at the 0.05 level of probability. The data were analysed with the statistical software SPSS version 20.0 (IBM Inc., New York, NY, USA). Stomatal characteristics and the characteristics of the leaves and roots were determined by ImageJ (http://rsb.info.nih.gov/ij/). The results are presented as the means ± SDs.

Results

Shoot regeneration from leaf explants

We studied the effects of BA and NAA on adventitious shoot regeneration from leaf explants of poplar 84 K under 9 different treatments (Table 1). Adventitious buds and calli formed from leaf explants after 20 days of culture (Fig. 1a), and shoots regenerated from leaf explants after 40 days (Fig. 1b). Both the shoot formation rate and the number of shoots per explant were affected significantly by BA ($F = 16.498$, $P = 0.000$) and NAA ($F = 16.777$, $P = 0.000$) concentration. Furthermore, the interaction between BA and NAA also significantly affected these parameters ($F = 5.581$, $P = 0.004$). When the explants were cultured on medium containing 0.50 mg L^{-1} BA and 0.05 mg L^{-1} NAA or 0.70 mg L^{-1} BA and 0.07 mg L^{-1} NAA, the shoot formation rate reached more than 90%. But the number of shoots per explant was higher on media containing 0.50 mg L^{-1} BA and 0.05 mg L^{-1} NAA than on media containing 0.70 mg L^{-1} BA and 0.07 mg L^{-1} NAA (Table 1). In general, media

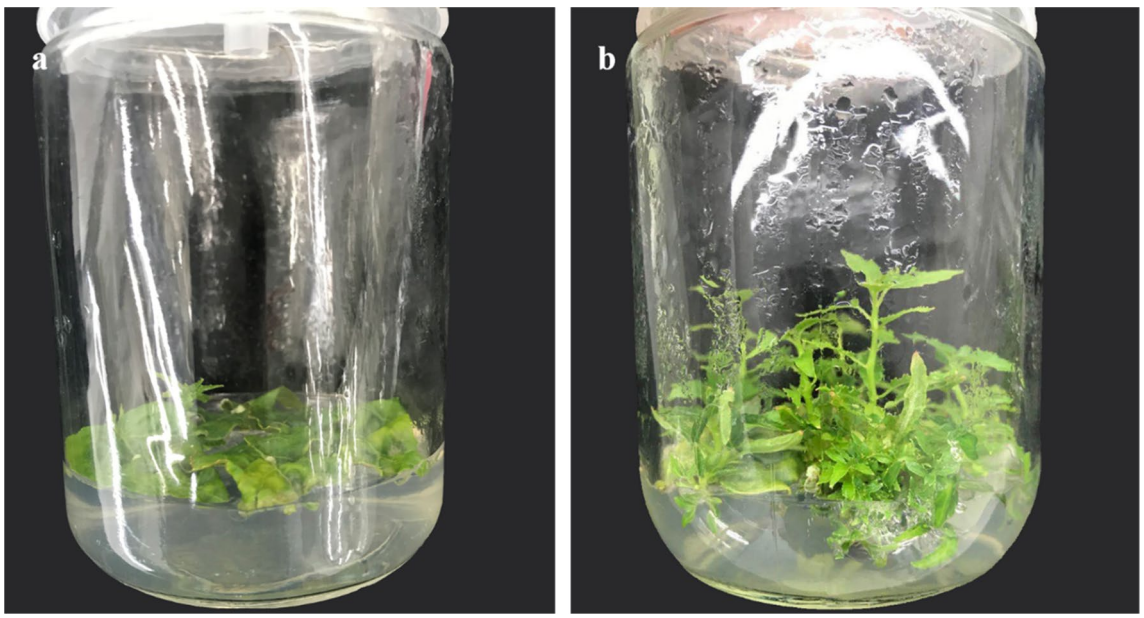

Fig. 1 Plant regeneration from leaf explants of the poplar 84 K. **a** Callus formed from leaf explants after 15 days of culturing, **b** shoots regenerated from leaf explants after 30 days

consisting of 0.50 mg L^{-1} BA and 0.05 mg L^{-1} NAA was the best media for shoot formation in the experiment.

Tetraploid induction by colchicine treatment and ploidy determination

Diploid polar 84 K was subjected to 27 different treatments (Table 2), we obtained 1490 regenerated shoots in total. Flow cytometry analysis revealed 98 tetraploids (Fig. 2). According to the chromosome counting method, we determined that the chromosome number of the diploid plants was 2n = 2× = 38 (Fig. 3a) and that the chromosome number of tetraploid plants was 2n = 4× = 76 (Fig. 3b).

The tetraploid induction rates derived from 27 treatments in poplar 84 K are presented in Table 2. As seen from the table, the tetraploid induction rates varied from 0 to 37.03%. The results indicated that the days of pre-culture (F = 25.187, p = 0.000), colchicine concentration (F = 20.889, p = 0.000) and exposure duration (F = 44.149, p = 0.000) caused significant differences in the induction rate of tetraploids. In addition, the frequency of tetraploid induction was significantly affected by the interaction among pre-culture duration, colchicine concentration and exposure duration, suggesting that specific combinations of the three analysed factors are necessary for in vitro tetraploid induction in poplar 84 K (F = 13.147, p = 0.000). In general, When the pre-culture duration and of exposure time were constant, the number of regenerated shoots decreased with the increase of colchicine concentration. When the pre-culture duration and colchicine concentration were constant, the induction rate of tetraploid decreased with the increase of exposure time. The highest tetraploid induction efficiency was 37.03%, which was achieved by treating leaves with 50 mg L^{-1} colchicine for 3 days after 4 days of pre-culture (Table 2).

Table 2 Effects of pre-culture duration, colchicine concentration and exposure time on tetraploid induction in poplar 84 K

Treatment	Pre-culture duration (d)	Colchicine concentration (mg L^{-1})	Exposure time (d)	No.of shoots regenerated[a]	No. of tatraploid[b]	Tetraploid induction rates (%)[c]
1	3	40	2	105	6	5.82 ± 0.80
2	3	40	3	79	3	3.83 ± 0.31
3	3	40	4	80	2	2.17 ± 1.56
4	3	50	2	66	4	6.09 ± 2.15
5	3	50	3	52	2	3.70 ± 2.62
6	3	50	4	43	0	0.00 ± 0.00
7	3	60	2	55	4	7.34 ± 2.69
8	3	60	3	60	3	5.25 ± 1.18
9	3	60	4	49	0	0.00 ± 0.00
10	4	40	2	73	6	8.31 ± 0.89
11	4	40	3	66	3	4.60 ± 0.47
12	4	40	4	66	2	3.03 ± 2.18
13	4	50	2	52	7	14.97 ± 4.93
14	4	50	3	44	16	37.03 ± 3.95
15	4	50	4	61	5	8.25 ± 2.07
16	4	60	2	60	5	8.23 ± 1.90
17	4	60	3	60	2	3.33 ± 2.36
18	4	60	4	54	0	0.00 ± 0.00
19	5	40	2	65	5	8.43 ± 3.36
20	5	40	3	54	2	4.33 ± 3.07
21	5	40	4	58	2	3.02 ± 2.14
22	5	50	2	37	7	18.07 ± 5.70
23	5	50	3	37	2	5.01 ± 3.61
24	5	50	4	32	0	0.00 ± 0.00
25	5	60	2	33	5	15.50 ± 3.30
26	5	60	3	22	3	13.90 ± 1.98
27	5	60	4	27	2	6.67 ± 4.71
Total				1490	98	

[a,b]Data represent the sum of three replicates

[c]Data represent the mean ± SD of three replicates

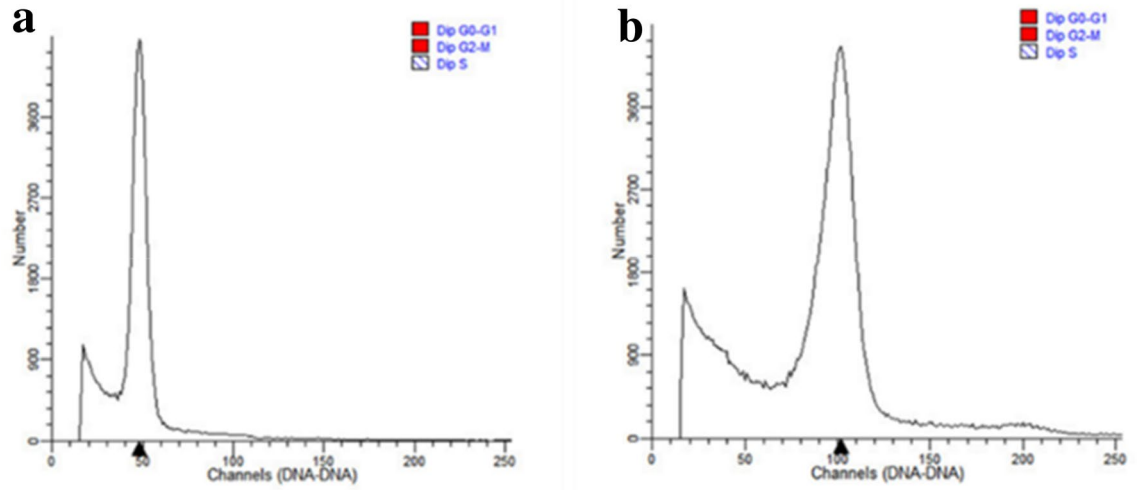

Fig. 2 Histograms of flow cytometric analysis of poplar 84 K (*Populus alba*×*P. glandulosa*). **a** Diploid plant, **b** induced tetraploid plant

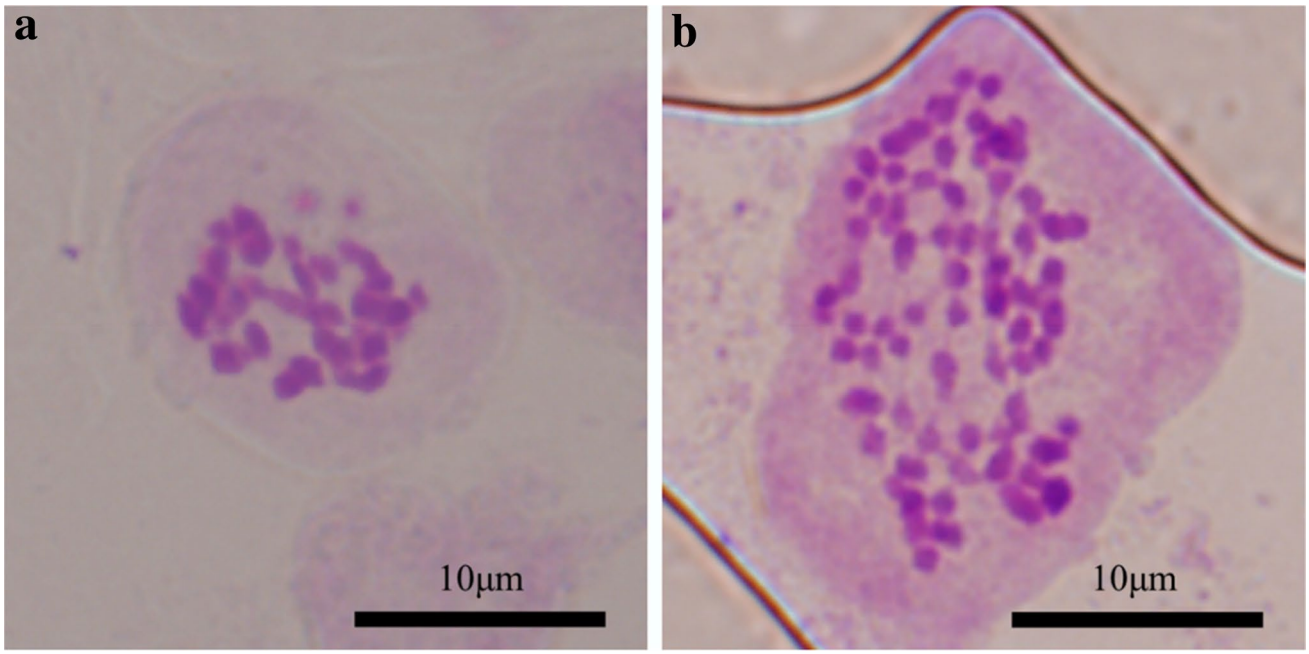

Fig. 3 Chromosomes of regenerated plants of poplar 84 K (*Populus alba*×*P. glandulosa*). **a** Diploid 2n = 2× = 38, **b** tetraploid 2n = 4× = 76. Bars = 10 μm

Differences in morphological features between tetraploid and diploid plants

To analyse the morphological changes associated with changes in ploidy level, we compared the stomatal length and width and stomatal density between tetraploid and diploid plants. By comparison, we found that there were significant differences between diploid and tetraploid plants in these parameters. The average stomatal length and width of the tetraploid plants were 19.02 μm and 9.00 μm, respectively, but the same parameters were only 8.48 μm and 4.50 μm for the diploids, respectively (Table 3). Compared with those of the diploid leaves, the stomata on the abaxial side of the tetraploid leaves were approximately 50% larger and half as dense (Figs. 4, 5). Moreover, the average diameter of the diploid protoplasts was 15.15 μm, which was smaller than that of the tetraploid protoplasts (25.79 μm) (Table 3).

In addition, there were phenotypic changes resulting from polyploidy. Root formation occurred on regenerated shoots

Table 3 Effect of ploidy level on Characteristics of poplar 84 K

Characteristics	Diploid	Tetraploid
Stomata length(μm)	8.48 ± 1.89b	19.02 ± 4.88a
Stomata width(μm)	4.50 ± 1.48b	9.00 ± 2.27a
Stomata density(mm^2)	117.65 ± 17.27a	57.00 ± 15.20b
leaf mesophyll protoplast diameter	15.15 ± 2.37	25.79 ± 4.25
Root length(cm)	8.82 ± 2.35	5.84 ± 1.26
No. of the root	4.38 ± 1.00	2.69 ± 0.72
Plant height(cm)	7.70 ± 1.10	5.49 ± 1.20

Each value represents the mean ± SD of two experiments that involved at least ten replicates. Values within the same column followed by different lower-case letters are significantly different based on a two-sample *t* test ($P < 0.05$)

on transfer to ½ MS medium containing 0.05 mg L^{-1} IBA and 0.02 mg L^{-1} NAA (Fig. 6). The length of the roots of the tetraploid plants was shorter than that of the diploid plants, and the tetraploid plants presented fewer roots per plant than did the diploid plants (Table 3). In addition, the plant height (Fig. 7a) was lower for the 30-day-old tetraploids than for the diploids of the same age (Table 3). Compared with the diploid leaves, the tetraploid leaves were also larger and more serrated (Fig. 7b).

Discussion

Colchicine is an alkaloid extracted from meadow saffron (*Colchicum autumnale* L.) and is the most widely used antimitotic agent for polyploidy induction (Planchais et al. 2000). Colchicine inhibits chromosome separation during cell division, resulting in chromosome doubling (Wu et al. 2020). Consequently, colchicine has become a common mutagen used in polyploid breeding (Blakeslee and Avery 1937). Numerous tetraploid plant species have been successfully induced by colchicine (Dermen and Henry 1944; Van et al. 1992; Tosca et al. 1995; Dhooghe et al. 2010). However, not all concentrations of colchicine are applicable for producing tetraploids. Low colchicine concentrations or short exposure times are ineffective for inducing somatic chromosome doubling (Allum et al. 2007). In addition, studies have shown that tetraploid induction frequency depends on the interaction between duration and concentration of mitotic inhibitors (Nilanthi et al. 2009; Cai and Kang, 2011). However, high colchicine concentrations or long exposure times are also ineffective because the toxicity of colchicine can lead to death of the tissue (Morejohn et al. 1987; Shao et al. 2003; Wu et al.2020). Therefore, appropriate colchicine concentrations and durations are keys to inducing tetraploids. Xu et al. (2016) found that 30 mg L^{-1} colchicine applied to explants was the most effective for inducing polyploidization [(*P. pseudo-simonii* × *P. nigra* 'Zheyin #3′) × (*P.* × *beijingensis*)]. In this study, we obtained a total of 98 tetraploids, which were achieved by treating leaves with 50 mg L^{-1} colchicine for 3 days. The results showed that colchicine treatment was effective at inducing tetraploidy in poplar 84 K.

Mixoploids are easily produced from somatic chromosome doubling of multi-cellular organs (Thao et al. 2003; Campos et al. 2009; Ewald et al. 2009; Cai and Kang, 2011; Xu et al. 2018; Liu et al. 2018). Xu et al. (2018) reported that the leaf incision callus developmental status of *Populus* affects tetraploid production efficiency, which also shows the importance of pre-cultivation. Here, the highest tetraploid induction efficiency was 37.03%, which was achieved by treating leaves with 50 mg L^{-1} colchicine for 3 days after 4 days of pre-culture. Our findings showed that the tetraploid

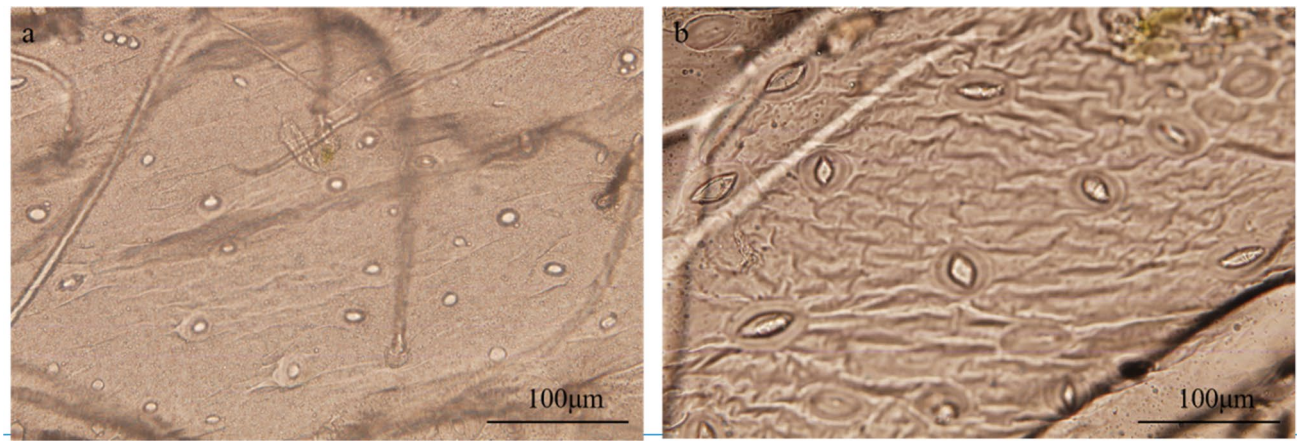

Fig. 4 Comparisons of stomata frequency and size of poplar 84 K (*Populus alba* × *P. glandulosa*) diploid and tetraploid. **a** Stomata of diploid plant leaf, **b** stomata of tetraploid plant leaf. Bars = 100 μm

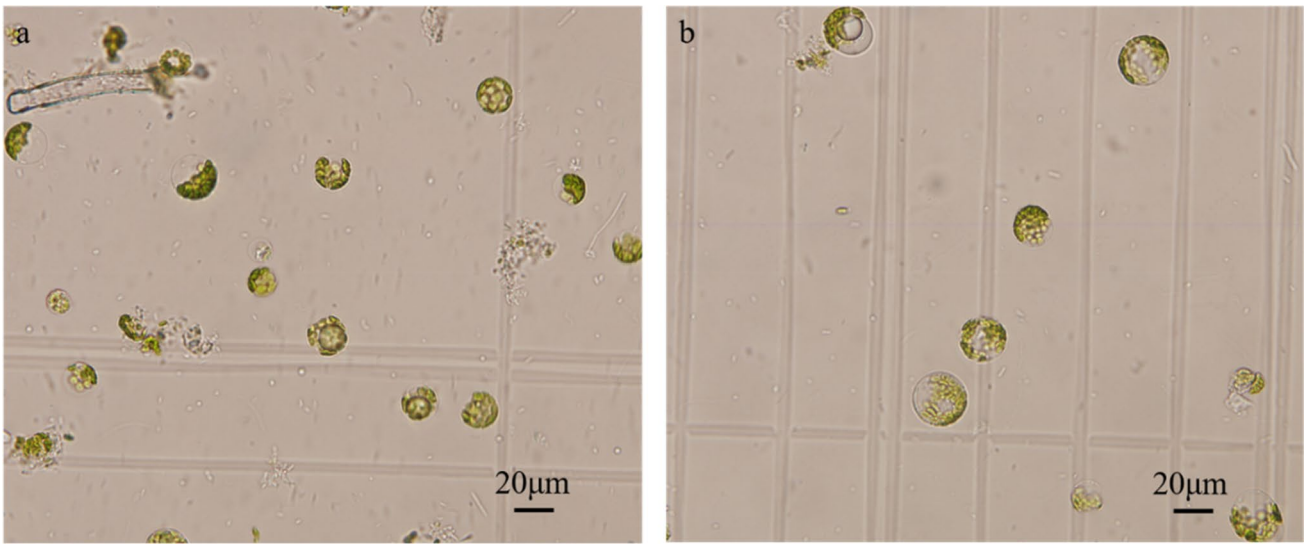

Fig. 5 Difference in the size of leaf mesophyll protoplasts between diploid and tetraploid poplar plants. **a** Leaf mesophyll protoplasts in the diploids, **b** leaf mesophyll protoplasts in the tetraploids. Bars = 20 μm

induction rate was dependent on interactions among preculture duration, concentration and duration of colchicine treatment. The findings are concomitant with the results reported by Xie et al. (2015) and Xu et al. (2016). According to another report, mixoploids are considered unstable due to asynchronism in the cell cycle between two types of cells (Dermen and Henry 1944; Mergen and Lester 1971; Wan et al. 1989; Nilanthi et al. 2009). However, several studies have reported the discovery of stable high-performance mixoploids in *Populus* (Ulrich and Ewald 2014; Xu et al. 2016; Wu et al 2020). In this study, a few mixoploids were also obtained (data not shown). They were stable for six months in subculture, showing that normal and polyploidized cells stably existed in the mixoploid cell cycles. The stability of these hybrids over a long period of time still needs to be studied.

Stomatal characteristics are considered a simple and useful method to determine ploidy level. Changes in stomatal size and density are common among tetraploids (Ascough et al. 2008; Sakhanokho et al. 2009; Rêgo et al. 2011; Talebi et al. 2017). Compared with diploids of *Populus*, tetraploids of *Populus* have a lower stomatal density and stomatal guard cells with a larger length and diameter (Lu et al. 2013). In this study, compared with those of the diploids, the stomata of the tetraploids were also approximately 50% larger (length and width) and less than half as dense (50%). Overall, these data suggest that stomatal size and density are reliable phenotypic markers for poplar 84 K polyploids.

Studies have revealed that polyploidy induces distinct phenotypic and morphological changes (Schranz and Osborn.2000) such as differences in leaf size and root architecture as well as alterations in plant physiology, biotic stress tolerance and other developmental processes (Cohen et al. 2013). Polyploidy often causes morphological features and growth development that are distinctly different from those of diploids (Stebbins 1950; Stanys et al. 2006; Allario et al. 2011; Dhooghe et al. 2010; Sattler et al. 2016). Compared with *Betula platyphylla* diploids, *Betula platyphylla* tetraploids are generally superior in terms of volume, leaves, fruit and stomata but are inferior in terms of height (Mu et al. 2012). Compared with *Ziziphus* diploids, *Ziziphus* tetraploids have larger and thicker leaves, darker green leaves, and shorter internodes (Gu et al. 2005; Cui et al. 2017). In *Paulownia australis* tetraploid plants, the leaf length and width are greater, the leaf size is larger, and the leaf structures are thicker, including the upper and lower epidermal layers and palisade tissues; however, the spongy parenchyma layer is thinner (Wang et al. 2017). In this study, compared with the diploid plants, the tetraploid plants had larger, thicker and more serrated leaves; larger but sparser stomata; fewer and shorter roots; and larger protoplasts. Poplar has a long growth cycle, so economic traits such as wood yield could not be analyzed in the present study. However, with the increase in ploidy, the morphological changes are obvious and could be used to study trait variations in polyploid poplar germplasm in field conditions. We expect that the finding of the present study could be applied for the production of novel germplasm for *Populus* breeding efforts.

Roots not only provide structural support to the aerial portion of plants but also acquire nutrients and water, which are vital to plant growth. Plant roots, especially lateral roots (LRs), play crucial roles in adaptation to various conditions (Casimiro et al. 2003; Nibau et al. 2008; Petricka et al. 2012; Lavenus et al. 2013). In this study, the length of the roots

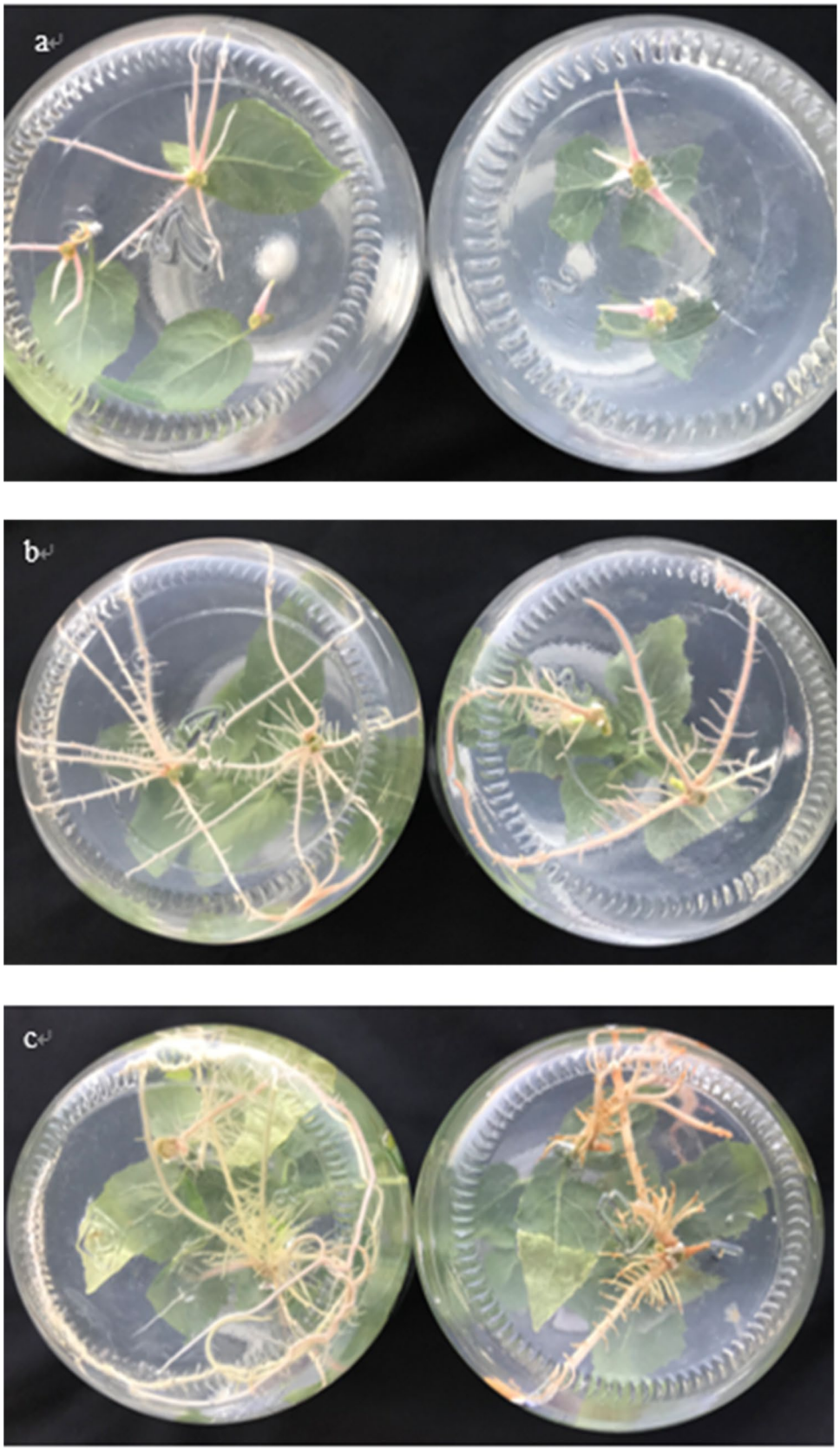

Fig. 6 Rooting of regenerated shoots cultured on ½ MS medium containing 0.05 mg L^{-1} IBA and 0.02 mg L^{-1} NAA. **a** Diploid (left) and tetraploid (right) shoots cultured on the rooting medium for 10 days, **b** diploid (left) and tetraploid (right)shoots cultured on the rooting medium for 25 days, **c** diploid (left) and tetraploid (right)shoots cultured on the rooting medium for 35 days

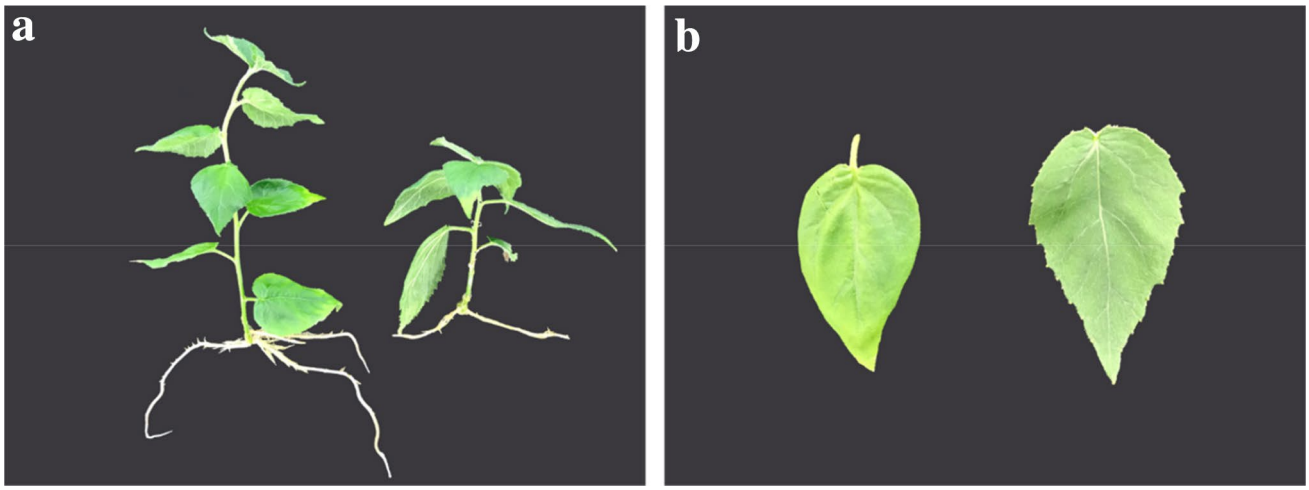

Fig. 7 Differences in morphological features of poplar 84 K (*Populus alba*×*P. glandulosa*). **a** Diploid (left) and induced tetraploid plants (right), **b** fully expanded leaf blades of diploid (left) and tetraploid (right)

of tetraploid plants was shorter than that of diploid plants, and the tetraploid plants presented fewer roots per plant than did the diploid plants, similar to the results reported for induced tetraploids of *Thymus persicus* (Tavan et al. 2015) and *Punica granatum* (Shao et al. 2003). Molecular mechanisms involving transcription factors and regulations in production of plant hormone could be the reasons for these differences which need to be studied.

Importantly, poplar 84 K is easily accessible for genetic transformation, and this clone is widely used by scientists as a model for woody species in terms of transgenic experiments. Qiu et al. (2019) described a de novo assembly of the genome sequence of the hybrid poplar (*P. alba*×*P. glandulosa*) clone 84 K and identified two subgenomes via comparison of the genomes. The obtained tetraploids in the present study increase genomic resources and contribute to further analysis of gene function and comparative differences in genes across poplar plants of different ploidy levels.

Conclusion

We studied the effects of BA and NAA on adventitious shoot regeneration from leaf explants of poplar 84 K, media consisting of 0.5 mg L^{-1} BA and 0.05 mg L^{-1} NAA was the best media for shoot formation. The regeneration system was used for production of colchicine induced polyploids in poplar 84 K, the frequency of tetraploid induction was significantly affected by the interaction among pre-culture duration, colchicine concentration and exposure duration, the highest tetraploid induction efficiency was 37.03%, which was achieved by treating leaves with 50 mg L^{-1} colchicine for 3 days after 4 days of pre-culture. By comparison, we found that there were significant morphological differences between diploid and tetraploid plants in leaf size, protoplast size, stomatal and root parameters.

Author contributions XY and YY conceived and designed the research. YY conducted the experiments, analyzed data and wrote the manuscript. YC processed figures. All authors have read and agreed to the published version of the manuscript.

Funding This work was financially supported by National Natural Science Foundation of China (31530012).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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