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ORIGINAL ARTICLE

Inducing triploids and tetraploids with high temperatures in *Populus* sect. *Tacamahaca*

Liqin Guo[1,2] · Wenting Xu[1] · Yan Zhang[1] · Jinfeng Zhang[1] · Zunzheng Wei[3]

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Abstract

Key message **This study is the first to report that triploids and tetraploids have been successfully produced through embryo sac and zygotic embryo chromosome doubling with high temperatures in *P. simonii* Carr. and its hybrid.**

Abstract A new synthetic polyploid induced by hybridization with unreduced gametes and heterozygotic embryo chromosome doubling can effectively combine polyploidy and heterosis, which can provide two major breeding advantages. In *Populus*, successfully creating and cultivating new polyploid varieties have economic and ecological production value. This was the first successful study in which embryo sac and zygotic embryo chromosome doubling was induced using high temperatures to produce triploids and tetraploids in *Populus simonii* Carr. and its hybrid, *P. simonii* × *P. nigra* var. *Italica*, of *Populus* sect. *Tacamahaca*. The relationship between flower bud morphological characteristics (time after pollination) and female meiotic stage (embryo sac and zygotic embryo development) was established to guide the induction treatment period. In the resulting progeny, 37 triploids and 12 tetraploids were obtained and identified using flow cytometry. The optimal temperatures for embryo sac and zygotic embryo chromosome doubling were 38 and 41 °C, respectively. Cytogenetic analysis revealed that 66–72 h after pollination (HAP), a period characterized by a high proportion of one-nucleate and two-nucleate embryo sacs, was the optimal period for embryo sac chromosome doubling. For zygotic embryo chromosome doubling, 168 HAP was the optimal induction period, as there was a high proportion of two-cell and four-cell proembryos. The results indicate that inducing embryo sac and zygotic embryo chromosome doubling is an ideal method for producing polyploids. The methods for inducing polyploids and for evaluating ploidy and offspring with different ploidies and heterozygosity in this study will be useful for genetic research and *Populus* breeding programmes.

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L. Guo and W. Xu contributed equally to this work.

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✉ Jinfeng Zhang
zjf@bjfu.edu.cn

✉ Zunzheng Wei
weizunzheng@163.com

[1] National Engineering Laboratory for Tree Breeding, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration, College of Biological Science and Biotechnology, Beijing Forestry University, Beijing 100083, People's Republic of China

[2] State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and Cultivation, State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, People's Republic of China

[3] Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (North China), Ministry of Agriculture, Key Laboratory of Urban Agriculture (North), Ministry of Agriculture, Beijing Vegetable Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, People's Republic of China

Keywords *Populus simonii* Carr. · Triploid breeding · Tetraploid breeding · High temperature · Megagametogenesis

Introduction

Poplar (*Populus* spp.) is the model organism of xylophytes (woody plants), as it is economically and ecologically valuable, fast growing, can easily propagate, and has a small genome, making it important in the economic development and ecological protection of forestry (Bradshaw et al. 2000). Polyploid breeding programmes, particularly triploid breeding, have become one of the most powerful approaches for genetically improving poplars since the first natural triploid, *P. tremula* ($2n = 3x = 57$), was discovered, with its high growth performance and resistance (Müntzing 1936; Nilsson-Ehle 1936). Heterosis and polyploidy in triploid poplars offer numerous advantages over their diploid counterparts, including greater growth vigour, larger leaves, better timber quality, higher stress resistance, and lower fertility (Nilsson-Ehle 1936; Zhu et al. 1998; Liao et al. 2016).

In general, triploids are selected from natural habitats (Nilsson-Ehle 1936; Zhu et al. 1998) or obtained by artificially controlled hybridization by crossing diploids with triploids or tetraploids (Winton and Einspahr 1970; Harder et al. 1976; Zhu et al. 1995). However, these methods rely on infrequent events due to a lack of natural triploids and tetraploids and irregular meiosis in triploids. Another method involves using spontaneous or artificial diploid ($2n$) pollen to create triploids and variation in poplar breeding (Zhu et al. 1998; Kang et al. 1999; Zhang et al. 2004; Zhang et al. 2009). However, Kang and Zhu (1997) reported that $2n$ pollen had a lower chance of fertilizing female gametes in competition with normal pollen, resulting in a low incidence of triploids. Recently, the use of $2n$ female gametes has been shown to be more effective in producing triploid poplars (Li et al. 2008; Wang et al. 2010, 2012; Xi et al. 2012; Lu et al. 2013). However, the female gametophyte is located inside the ovule, which inhibits real-time observation and determination of the effective $2n$ female gamete induction treatment period. Therefore, observing megagametogenesis is a prerequisite for inducing $2n$ female gametes to clarify the relationship between flower bud morphological characteristics (time after pollination) and female meiotic stage (embryo sac and zygotic embryo development) and helps to determine the optimal induction treatment period.

Colchicine is effective for inducing $2n$ female gametes in poplars (Li et al. 2008; Wang et al. 2010; Xi et al. 2012), but procedures are complicated and time-consuming. Furthermore, high temperature, a physical mutagen, is effective in inducing polyploids in plants (Randolph 1932; Wang et al. 2013) and animals (Nomura et al. 2004; Yang and Guo 2006), and it allows for uniformity of treatments and has economic, procedural, and operational advantages. In poplars, Wang et al. (2012, 2013) successfully induced triploids and tetraploids in treated *P. pseudo-simonii* × *P. nigra* 'Zheyin3#' female catkins with high temperature exposure. In addition, Lu et al. (2013) also reported the induction of $2n$ female gametes in *P. adenopoda* during female gametophyte development treated with high temperature. Thus, high temperature exposure may be an appropriate method for poplar polyploid production.

Populus simonii Carr. belongs to *Populus* sect. *Tacamahaca*. This indigenous tree is widely used in northern China for its adaptability and drought, cold, and saline-alkaline resistances (Wei et al. 2013). However, there are few reports of polyploid breeding for *P. simonii*. Polyploid *Tacamahaca* trees should be more stress resistant and have faster timber growth, leading to increased wood production and increased value in terms of environmental protection and local economic development. Therefore, it is necessary to develop polyploid breeding in *Tacamahaca* poplars, especially *P. simonii*. Research on polyploid breeding and genetic improvement of *Tacamahaca* poplars could produce new varieties with rapid growth and high timber quality, which would also help to enrich evolutionary theory, economic development, and ecological protection. The objective of our research was to investigate the production of triploids and tetraploids using high temperatures during embryo sac and zygotic embryo development in *P. simonii* based on cytological analysis of female gametophyte and zygotic embryo development. The methods for inducing polyploids and evaluating ploidy in this study could have practical application value in polyploid breeding in *Populus*.

Materials and methods

Plant material

Healthy floral branches of a male *P. simonii* × (*P. pyramidalis* + *Salix matsudana*) clone ($2n = 2x = 38$) parent and two female *P. simonii* and *P. simonii* × *P. nigra* var. *Italica* ($2n = 2x = 38$) parents were collected from a plantation in Tongliao City (Inner Mongolia, People's Republic of China). Floral branches of another male *P. simonii* × (*P. pyramidalis* + *S. matsudana*) ($2n = 2x = 38$) parent were collected from a plantation in Dadongliu (Beijing, People's Republic of China). Robust floral branches with plump flower sprouts were obtained and carried back to a greenhouse at Beijing Forestry

University with plastic film as packaging. Next, the two male parent floral branches were trimmed and cultured in a bucket with water to force flower development in the greenhouse (10–20 °C). Approximately 1 week later, the two female parent floral branches were trimmed and cultured with same culture condition (Fig. S1a).

Cytological analysis of megagametogenesis and zygotic embryo development

To investigate the developmental process of *P. simonii* megagametogenesis and zygotic embryos, female catkins were sampled at different periods for observation. After the branches were cultured in the greenhouse, female catkins were collected at 09:00 and 15:00 daily until pollination. After pollination, female catkins were collected at 12 h intervals for 72 h and then collected every 24 h. Before fixation, the morphological characteristics of the catkins were recorded using photography. The collected samples were fixed immediately in formalin-acetic alcohol (FAA) (18:1:1, 70% ethanol: acetic acid: 40% formaldehyde) at 4 °C for 24 h and then stored in 70% ethanol. Subsequently, the ovaries from each fixed bud were dehydrated with a graded ethanol series and embedded in paraffin. Serial longitudinal sections (4 μm) were cut with a rotary microtome, fixed on slides, and stained with 0.5% haematoxylin. The preparations were observed under an Olympus BX43 microscope and photographed using an Olympus DP73 camera system. At least four catkins were sampled and 30 capsules were randomly observed for each specimen. The numbers of ovules at different development stages were counted, and the percentage of each ovule type was calculated.

Treatment with high temperatures

In the process of water cultivation, the water in the bucket was changed every 3 days, which can effectively guarantee water absorption in the branches. Artificially controlled multiple hybridization pollination was performed in a greenhouse using the twig and pot water culture (tap water culture) method (Mofidabadi et al. 1998) in the spring. Before anther dehiscence, tracing paper was used to wrap the catkin to collect the pollen (Fig. S1b). After the pollen dispersal period, the collected pollen was transferred to different vials and then stored at 4 °C in a refrigerator after silica gel desiccant was added for later use. When the stigmas were at the receptive stage, the different pollen was poured into different petri dishes (Fig. S1c), and the pollen was painted on the stigmas of female catkins gently with different brushes according to the hybridized combination (Fig. S1d). We were careful not to mix different pollen during the whole pollination process.

To double embryo sac chromosomes, female branches were exposed to 38, 41, and 44 °C for 2, 4, and 6 h at 66, 72, and 78 h after pollination (HAP) following an orthogonal experimental design L_9 (3^4). To double zygotic embryo chromosomes, female branches were exposed to 38, 41, and 44 °C for 2, 4, and 6 h at 120, 144, and 168 HAP following an orthogonal experimental design L_9 (3^4). The high temperature treatment was carried out in a phytotron where the temperatures could be controlled (Fig. S1e). Untreated branches served as the control group. Each treatment gradient including the control group had five selected female branches, and each branch kept five catkins after pollination and continued water cultivation.

Seeds matured after approximately 30 days of cultivation. Before capsule (Fig. S1f) dehiscing, each catkin was bagged to collect seeds, and the different hybrid combinations, treatment conditions, and collection times were recorded (Fig. S1g). After all capsules dehisced, the catkins were removed together with the bag (Fig. S1h). According to collection time, the seeds were picked out manually and then put into vials containing silica gel desiccant (Fig. S1i) before being stored at 4 °C in a refrigerator. The seeds were germinated in a sterile mixed medium (Fig. S1j) which contained soil, vermiculite and peat (5:1:1, v/v) and placed in a phytotron where the temperature, humidity, and illumination time was controlled for growing (Fig. S1k). The seedlings were transferred into containers with nutrient-supplemented soil (soil:vermiculite:peat:perlite = 2:1:1:1, v/v) after reaching 5 cm in height (Fig. S1l). When the surviving seedlings were approximately 30 cm in height, they were transplanted in the field.

Ploidy determination by stomatal characteristic analysis and flow cytometry

The preliminary polyploidy determination was conducted on all hybrid offspring by examining stomatal characteristics. The lower epidermis of mature leaves (two or three leaves down from the top of the plant) was removed using tweezers, placed on a glass slide with a drop of water, and flattened into the water drop with a dissecting needle. The preparations were observed under an Olympus BX43 microscope and photographed with an Olympus DP73 camera system. A chloroplast number per stomatal guard cell ≥13 was marked and recorded. The final polyploidy determination was conducted on the marked hybrid offspring using flow cytometry. Crude nuclei were prepared from 500 mg of fully expanded, field-grown leaves by chopping each sample for 30 s with a sharp razor blade in 1 ml of extraction buffer (Doležel et al. 1989). Another 1 ml of extraction buffer was added and gently blended for 2 min. The suspension was filtered using a 50-μm nylon filter, and the crude nuclei were separated from the filtrate

by centrifugation at 800 rpm for 5 min. The pellet was re-suspended in 1 ml of 50 μg ml^{-1} propidium iodide (PI)/Triton X-100 staining solution with RNase A (Robinson 2006) for at least 30 min in the dark. The samples were analysed using a FACSCalibur flow cytometer with an argon laser at 488 nm for PI excitation at 10,000 events. The mean nuclear DNA content of each sample was determined from the scanned nuclei. A leaf sample from a known diploid *P. simonii* specimen was used as a control. For each putative polyploid, samples were independently characterized three times. The ploidy levels of the hybrid offspring were determined by comparing the DNA content of the sample with the internal diploid control.

Statistical analysis

The rates of triploid and tetraploid production were analysed using a range analysis and polyploid induction rate to reveal differences according to time after pollination, temperature, and treatment duration.

Results and analysis

Cytological analysis of megasporogenesis and its relationship with female flower bud morphogenesis

Megasporogenesis of all megasporocytes finished approximately 14.5 days after being cultured due to asynchrony in *P. simonii* (Tables 1, 2). More than one meiotic stage was observed in each female bud due to asynchronous megasporocyte development among the ovaries. According to the regulations for sampling, there were 21 stages in the samples before pollination, but only the last ten stages were representative stages in which megasporogenesis can been observed (Fig. 1).

At stage I, the catkin was tightly wrapped in the bract scales, and the stigma had no bulging (Fig. 1); at this point, all megasporocytes were in interphase (Fig. S2a; Table 1). At stage II, the bract scales were partially dehisced, and the stigma began to bulge (Fig. 1) and 21.05 and 15.78% of the megasporocytes were in leptotene and pachytene, respectively (Fig. S2b–d; Table 1). At stage III, the bract scales were fully dehisced, the catkin started to emerge, and the embryonic stigma form appeared (Fig. 1). Most megasporocytes in this stage were in prophase I, including 37.5, 25, and 14.58% in leptotene, pachytene, and diakinesis (Fig. S2f; Table 1), respectively, while 8.33% of the megasporocytes had entered metaphase I (Fig. S2g; Table 1). At stage IV, the catkin began to protrude from the bract scales, and the stigma had grown (Fig. 1). Most megasporocytes were in prophase I, while 4.65% were in metaphase I, and 2.33% had entered anaphase I (Fig. S2h–

Table 1 Statistics of megasporocyte meiotic stages by developmental stage in *P. simonii*

Female floral stage	Interphase (%)	Meiosis I (%)						Meiosis II (%)	One-nucleate embryo sac (%)	Two-nucleate embryo sac (%)	Four-nucleate embryo sac (%)	Eight-nucleate embryo sac (%)
		Prophase I (%)			Metaphase I (%)	Anaphase I (%)	Telophase I (%)					
		Leptotene	Pachytene	Diakinesis								
1	100											
2	63.17	21.05	15.78									
3	14.58	37.5	25	14.58	8.33							
4	13.95	23.26	37.21	18.60	4.65	2.33						
5	11.76	23.53	35.29	11.76	11.76		5.88					
6		23.90	28.26	21.74	4.35	4.35	4.35	8.70	4.35			
7		18.18	27.27	13.64	2.27	2.27	4.55	20.46	11.36			
8		7.32	17.07	19.51				19.51	17.07	12.20	7.32	
9		5.26	21.05	15.78		5.26		15.78	21.05	10.53	5.26	
10		6.67	13.33	26.67				6.67	20	13.33	6.67	6.67

Table 2 Statistics of embryo sac by developmental stage in *P. simonii* after pollination

Time after pollination (h)	Meiosis I (%)	Meiosis II (%)	One-nucleate embryo sac (%)	Two-nucleate embryo sac (%)	Four-nucleate embryo sac (%)	Eight-nucleate embryo sac and mature embryo sac (%)	Hypnozygote period		Two-celled proembryo (%)	Four-celled proembryo (%)
							Free endosperm nuclei (%)	Hypnozygote (%)		
0	10.71	35.71	21.43	17.86	7.14	7.14				
12	8.33	33.34	25.00	16.67	8.33	8.33				
24		26.93	34.61	23.08	7.69	7.69				
36		14.29	35.71	14.29	14.29	21.43				
48		7.69	38.46	19.21	15.38	15.38	3.84			
60			34.78	26.09	17.49	17.39	4.35			
72			25.54	29.78	19.15	17.02	8.51			
96				22.22	3.70	7.41	11.11	55.56		
120				11.76	8.82	5.88	29.41	38.23	5.88	
144							61.90	23.81	9.52	4.76
168							53.85	23.08	7.69	15.38

i; Table 1). At stage V, approximately one fifth to one fourth of the catkin had emerged from the bract scales (Fig. 1). Most megasporocytes had undergone leptotene and pachytene, and 11.76 and 5.88% had entered metaphase I and telophase I (Table 1), respectively. At stage VI, one third of the catkin had emerged from the bract scales, which were easily exfoliated (Fig. 1). Approximately 83.95% of the megasporocytes were undergoing the first meiotic division, 8.7% had entered the second meiotic division (Fig. S2k–p; Table 1), and 4.35% had developed into one-nucleate embryo sacs (Fig. S3a; Table 1). At stage VII, half of the catkin had emerged (Fig. 1), and the developmental characteristics of the megasporocytes were similar to those in stage VI (Table 1). At stage VIII, three fifths of the catkin had emerged (Fig. 1), and 12.20 and 7.32% of the megaspores had developed into two-nucleate and four-nucleate embryo sacs, respectively (Fig. S3b–d; Table 1). At stage IX, four fifths of the catkin had emerged (Fig. 1), and the developmental characteristics of megasporocytes were similar to those at stage VIII, with 21.05, 10.53, and 5.26% of megaspores in the one-nucleate, two-nucleate, and four-nucleate embryo sac stages, respectively (Table 1). At stage X, nearly the entire catkin had emerged from the bract scales, and the stigma had opened slightly (Fig. 1), entering the receptive pollination period. At this stage, 6.67% of the megaspores had developed into eight-nucleate embryo sacs (Fig. S3e–f; Table 1).

Conventional cytological analysis revealed asynchronous meiosis in *P. simonii*, especially from stages VIII to X. However, at the early developmental stages, asynchrony and the distribution range of meiosis were not obvious in megasporocytes. Furthermore, certain meiotic stages were more prevalent in certain developmental periods, which could indicate an effective treatment period for inducing 2*n* female gametes. Thus, there was a correlation between megasporocyte meiosis and female flower

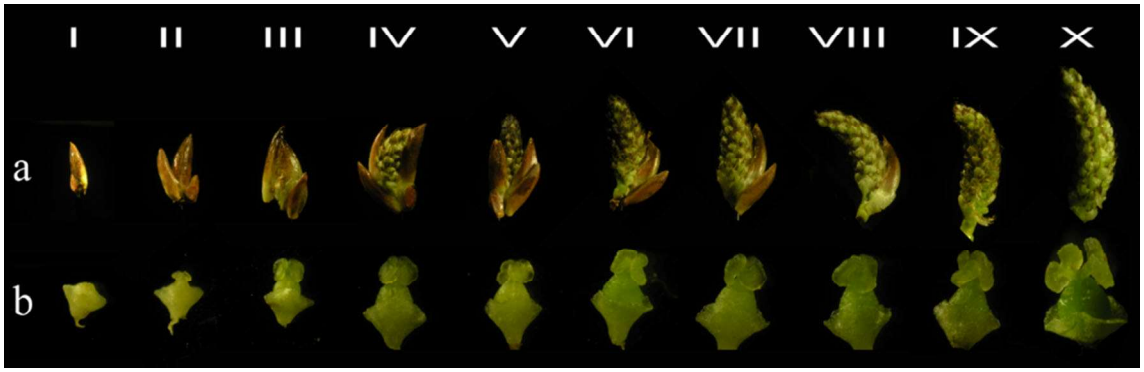


Fig. 1 Morphological characteristics of female flower development before pollination in *P. simonii*. **a** Female bud development, **b** capsule and stigma development

bud morphology at the early developmental stages, before one third of the catkin protruded from the bract scales. It was possible to determine meiosis of megasporocytes based on the morphological characteristics of female flower buds, which could increase the efficiency of chromosome doubling of megasporocytes in *P. simonii*.

Cytological analysis of megagametogenesis and zygotic embryo development and its relationship with time after pollination

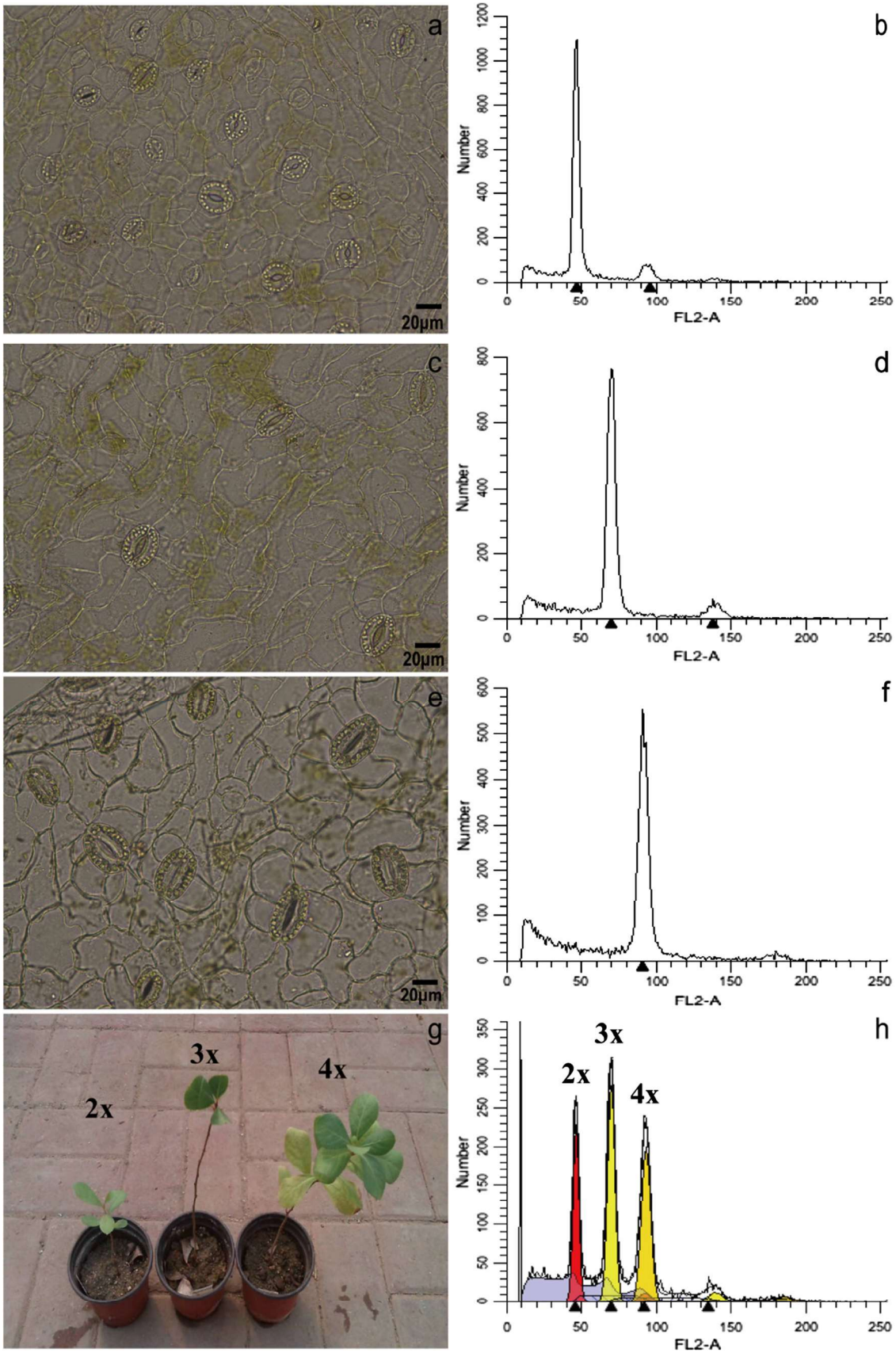
At 0 HAP, 10.71 and 35.71% of the megasporocytes were in meiosis I and II, respectively (Table 2). At 12 HAP, 33.34% of the megasporocytes were in meiosis II, but 8.33% of the megasporocytes were still in meiosis I. Meanwhile, 25.00, 16.67, and 8.33% of the megaspores had developed into one-nucleate, two-nucleate, and four-nucleate embryo sacs, respectively (Fig. S3a–d), and some megaspores had matured to eight-nucleate embryo sacs (Fig. S3g–h). At 24 HAP, all megasporocytes had completed meiosis I, and 26.93% were in meiosis II (Table 2). Most of the other megaspores had developed into one-nucleate and two-nucleate embryo sacs (Fig. S3a, b). At 36 HAP, most megaspores had become one-nucleate embryo sacs, although 14.29% of the megasporocytes were still in meiosis II. At 48 HAP, most megaspores had become one-nucleate embryo sacs, although 7.69% megasporocytes were still in meiosis II (Table 2). In addition, 3.84% of the megaspores had completed megagametogenesis, and post-fertilization zygote cells entered the free endosperm nuclear period (Fig. S4a–g). At 60 HAP, all megasporocytes had completed meiosis, and most megaspores were one-nucleate and two-nucleate embryo sacs (Table 2). At 72 HAP, the megaspore developmental characteristics were similar to those at 60 HAP (Table 2). At 96 HAP, all megaspores had completed the first mitosis, and 55.56% of post-fertilization zygote cells were in the hypnozygote period (Fig. S4h). At 120 HAP, most zygote cells were in the hypnozygote and free endosperm periods (Table 2), and 5.88% of hypnozygotes had become two-celled proembryos (Fig. S4i). By 144–168 HAP, all megaspores had completed megagametogenesis, and some hypnozygotes had become four-celled proembryos (Fig. S4j).

Overall, *P. simonii* had monosporic and initially seven-celled/eight-nucleate *Polygonum*-type embryo sacs. The functional megaspore underwent three successive mitotic divisions to produce an eight-nucleate megagametophyte. Likewise, conventional cytological analysis revealed asynchronous development after pollination in *P. simonii,* especially from 0 to 48 HAP. Some female gametophytes were even observed in the first meiotic division at 0–12 HAP. However, most other female gametophytes observed were finished with meiotic division and had developed into embryo sac at 0–12 HAP. All embryo sacs had completed double fertilization, and the zygotes had developed into hypnozygotes and free endosperm nuclei at 144 HAP.

Polyploid production

After female buds of *P. simonii* were treated with high temperatures, bud development was retarded, and the stigmas became dry and brown. Some buds died, and no seeds were collected for some treatments. In total, 8523 seeds were collected from the surviving treated buds. After sowing seeds and transferring young seedlings to the field, 2133 seedlings remained. From the stomatal characteristic analysis, when the chloroplast number of one stomatal guard cell was ≥13 (Fig. 2a, c, e), 453 hybrid offspring were marked as putative polyploids from the surviving seedlings. All 453 offspring were examined using flow cytometry, and 37 triploids induced by embryo sac chromosome doubling and 12 tetraploids induced by zygotic embryo chromosome doubling were screened (Fig. 2b, d, f, h) and tested three times for accuracy. All polyploids were from the treated groups. No polyploids were found in the control group.

Table 3 lists the yield and efficiency of triploid induction by treatment. Table 4 lists the range analysis results of the orthogonal experimental design of triploid induction in different parent combination (Xu and Li 2014; Yu et al. 2008). The K value for each level of a parameter is the summation of three values of experimental results (K_{jm} = summation of three values of j factor and m level), and the X value for each level of a parameter is the average of K value ($X_{jm} = K_{jm}/3$). The range value (R) for each factor is the difference between the maximum X value and minimum X value of the three levels. According to the principle of range analysis (Xu and Li 2014; Yu et al. 2008), a larger range value (R) means that the corresponding factor has a more significant influence in the experiment. For each factor, a larger X value means that the corresponding level was best and determines the optimal combination of factors. According to the range analyses and Table 3, the temperature has a more significant influence in the experiment. In addition, the treatment at 38 °C for 2 h at 66 HAP had the best effect in the female parent *P. simonii* hybridized combination, where the highest frequency of triploid induction was 7.76%. For the female parent *P. simonii* × *P. nigra* var. *Italica*, the treatment at 38 °C for 4 h at 72 HAP had the best effect, where the highest rate of triploid induction was 11.76%. In total, 37 triploids were detected after treating female catkins with high temperatures in *P. simonii* and its hybrid, *P. simonii* × *P. nigra* var. *Italica*. Cytological observation (Table 2) revealed that the proportion of one-nucleate and two-nucleate embryo sacs was highest at 66 and 72 HAP,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◀**Fig. 2** Ploidy level of offspring induced with high temperature (**a**, **c**, **e** *bar* 20 μm). **a** Stomatal guard cells analysis of control diploid plant, **b** flow cytometry analysis of control diploid plant, **c** stomatal guard cells analysis of triploid plant, **d** flow cytometry analysis of triploid plant, **e** stomatal guard cells analysis of tetraploid plant, **f** flow cytometry analysis of tetraploid plant, **g** control diploid ($2n = 2x = 38$), triploid ($2n = 3x = 57$) and tetraploid ($2n = 4x = 76$) plants, **h** flow cytometry analysis of nuclei mixture of leaves from control diploid, triploid and tetraploid plants

respectively, suggesting that the first and second mitotic divisions in embryo sac development provided greater chances for embryo sac chromosome doubling induced by high temperatures.

Table 5 lists the yield and efficiency of tetraploid induction by treatment and indicated that the best combination of factors was the treatment at 41 °C for 2 h at 168 HAP, and the highest rate of tetraploid induction was 5.95%. In total, 12 tetraploids were detected after treating female catkins with high temperatures in *P. simonii* and its hybrid, *P. simonii* × *P. nigra* var. *Italica*. Cytological observation (Table 2) revealed the proportion of hypnozygotes was higher at 168 HAP, suggesting that the first mitotic divisions in zygotic embryo development provided a greater chance for zygotic embryo chromosome doubling induced by high temperatures.

Discussion

Asynchrony of megagametogenesis and zygotic embryo development in *P. simonii*

For gamete and zygotic embryo chromosome doubling, the appropriate mutagen must be applied at the appropriate developmental stage (Kang et al. 1999). However, it was not possible to judge megagametogenesis and zygotic embryo development in real time, and observations were conducted in paraffin-embedded samples. Therefore, characterizing and comparing megagametogenesis and zygotic embryo development with flower bud morphological characteristics and time after pollination established a temporal relationship to guide mutagen treatment (Wang et al. 2010; Lu et al. 2013; Xi et al. 2014), which alleviated the workload and improved the efficiency of inducing chromosome doubling. A growing number of studies have shown that meiosis asynchrony is a ubiquitous phenomenon (Tavoletti 1994; Magnard et al. 2001; Risso-Pascotto et al. 2004; Li and Huang 2006). Studies on the female gametes and pollen of poplars, including *P. tomentosa* × *P. bolleana*, *P. adenopoda*, and *P.* × *euramericana*, among others, have observed asynchronous megasporogenesis and microsporogenesis in the same flower bud and floret and even in the same capsule and

Table 3 Results of triploid induction in embryo sacs treated with high temperature after pollination

	Time after pollination (h)	Temperature (°C)	Treatment duration (h)	*P. simonii* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)			*P. simonii* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*) *clone*			*P. simonii* × *P. nigra* var. *Italica* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)		
				No. of seedlings	No. of triploids	Rate of triploid production (%)	No. of seedlings	No. of triploids	Rate of triploid production (%)	No. of seedlings	No. of triploids	Rate of triploid production (%)
1	1 (66)	1 (38)	1 (2)	103	8	7.76	91	5	5.49	57	5	8.77
2	1 (66)	2 (41)	2 (4)	11	0	0	22	0	0	23	0	0
3	1 (66)	3 (44)	3 (6)	7	0	0	6	0	0	4	0	0
4	2 (72)	1 (38)	2 (4)	98	5	5.10	59	2	3.39	62	12	11.76
5	2 (72)	2 (41)	3 (6)	0	0	0	0	0	0	0	0	0
6	2 (72)	3 (44)	1 (2)	0	0	0	0	0	0	17	0	0
7	3 (78)	1 (38)	3 (6)	54	0	0	53	0	0	62	0	0
8	3 (78)	2 (41)	1 (2)	37	0	0	28	0	0	27	0	0
9	3 (78)	3 (44)	2 (4)	3	0	0	0	0	0	0	0	0

Table 4 The range analysis results of triploid induction

	P. simonii × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)			*P. simonii* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*) *clone*			*P. simonii* × *P. nigra* var. *Italica* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)		
	Time after pollination (h)	Temperature (°C)	Treatment duration (h)	Time after pollination (h)	Temperature (°C)	Treatment duration (h)	Time after pollination (h)	Temperature (°C)	Treatment duration (h)
K1	8	13	8	5	7	5	5	17	5
K2	5	0	5	2	0	2	12	0	12
K3	0	0	0	0	0	0	0	0	0
X1	2.67	4.33	2.67	1.67	2.33	1.67	2.67	5.67	2.67
X2	1.67	0	1.67	0.67	0	0.67	4	0	4
X3	0	0	0	0	0	0	0	0	0
Range	2.67	4.33	2.67	1.67	2.33	1.67	4	5.67	4

K value: the summation of three values of experimental results (K_{jm} = summation of three values of *j* factor and *m* level). *X* value: the average of *K* value ($X_{jm} = K_{jm}/3$). Range value (*R*): the difference between the maximum *X* value and minimum *X* value of the three levels

anther (Kang et al. 2000; Wang et al. 2006; Xi et al. 2012; Lu et al. 2013). In this study, asynchronous megasporogenesis was revealed in *P. simonii* through conventional cytological analyses. Meiosis was observed in megasporocytes with different morphological characteristics using paraffin-embedded samples, which showed that meiosis of *P. simonii* megasporocytes was asynchronous. Similar to megasporocyte meiosis, embryo sac and zygotic embryo development was asynchronous.

The asynchronous development is important in population reproduction and propagation. In plants, asynchrony is a characteristic of male and female gametophyte development. This evolutionary adaptation of the natural environment can extend the effective fertilization time between male and female flowers and promote population reproduction and propagation (Wyatt 1982; Harder and Thomson 1989).

An efficiently combined method to determine the ploidy of seedlings in large groups

In poplars, there are many methods to identify ploidy level. The most accurate is conventional chromosome counting at the root tip, but this method is laborious and time-consuming. It is difficult to obtain clear microscopy images without chromosome overlap because of the large number and small size of poplar chromosomes (Kang 1996). Wang et al. (2008) found a correlation between chromocentre number and ploidy level, and counting chromocentres in interphase nuclei provided a preliminary assessment of plant ploidy, which would provide a new method for identifying polyploids in poplars. In poplar polyploid breeding studies, using chloroplast number in epidermal stomatal guard cells has been confirmed in sections *Tacamahaca* and *Leuce Duby* (Cai and Kang 2011; Xi et al. 2012; Lu et al. 2013; Wang et al. 2013). This method is relatively simple, accurate, and efficient, and it can be used at the seedling stage, which can save on labour and resources. Because this characteristic is necessary for polyploids but not sufficient for identification, detection accuracy is low. Recently, flow cytometry has been shown to be a useful method in poplars because it is faster and more convenient for analysing nuclear DNA ploidy and has high detection accuracy (Bradshaw and Stettler 1993, 2009; Ewald et al. 2009; Xi et al. 2012; Lu et al. 2013; Wang et al. 2013). Therefore, in this study, stomata size and density and chloroplast number in epidermal stomatal guard cells are the best preliminary screening methods for polyploidy, especially for a large number of offspring seedlings. Afterwards, flow cytometry can be conducted to identify polyploidy.

Induction of chromosome doubling in embryo sacs and zygotic embryos

High temperatures can inhibit chromosome separation during embryo sac mitosis and induce embryo sac chromosome doubling. In a polyploid breeding programme that induced embryo sac chromosome doubling in *P. pseudo-simonii* × *P. nigra* 'Zheyin3#' (Wang et al. 2012), treatments at 41–44 °C were the most suitable temperatures for embryo sac chromosome doubling. Moreover, Wang et al. (2012) reported that the increased frequency of eight-nucleate embryo sacs suggested that the third mitosis during embryo sac development could be the optimal stage for high temperature-induced triploid production. Furthermore,

Table 5 Results of polyploid induction in zygotic embryos treated with high temperature after pollination

	Time after pollination (h)	Temperature (°C)	Treatment duration (h)	*P. simonii* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)			*P. simonii* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*) *clone*			*P. simonii* × *P. nigra* var. *Italica* × *P. simonii* × (*P. pyramidalis* + *S. matsudana*)		
				No. of seedlings	No. of tetraploids	Tetraploid production rate (%)	No. of seedlings	No. of tetraploids	Tetraploid production rate (%)	No. of seedlings	No. of tetraploids	Tetraploid production rate (%)
1	1 (120)	1 (38)	1 (2)	77	0	0	87	0	0	26	0	0
2	1 (120)	2 (41)	2 (4)	0	0	0	99	0	0	0	0	0
3	1 (120)	3 (44)	3 (6)	0	0	0	0	0	0	0	0	0
4	2 (144)	1 (38)	2 (4)	111	0	0	103	0	0	74	0	0
5	2 (144)	2 (41)	3 (6)	0	0	0	0	0	0	0	0	0
6	2 (144)	3 (44)	1 (2)	0	0	0	0	0	0	67	0	0
7	3 (168)	1 (38)	3 (6)	121	0	0	127	0	0	0	0	0
8	3 (168)	2 (41)	1 (2)	151	5	3.31	110	2	1.82	121	5	5.95
9	3 (168)	3 (44)	2 (4)	0	0	0	0	0	0	35	0	0

in *P. adenopoda* (Lu et al. 2013), the best conditions for embryo sac chromosome doubling using high temperatures was 38 °C for 6 h at 18 HAP. In addition, in *P. adenopoda*, the two-nucleate embryo sac may be the most effective stage to induce 2*n* female gametes, and the second mitosis stage may also be more effective. In this study, the optimal treatments were 38 °C for 2–4 h at 66–72 HAP, and the proportion of one-nucleate and two-nucleate embryo sacs was highest at 66 and 72 HAP, respectively, suggesting that the first and second mitotic divisions in embryo sac development provided the greatest opportunities for embryo sac chromosome doubling induced by high temperatures in *P. simonii*. The differences between this study and previous research could be due to differences in organization structure and embryo sac development, as well as greater temperature sensitivity in *P. simonii*. The triploid proportion induced by embryo sac chromosome doubling in *P. tomentosa* × *P. bolleana* (Kang et al. 2004) and *P. pseudo-simonii* × *P. nigra* 'Zheyin3#' (Wang et al. 2010) was more than 50%, and the highest efficiency of triploid induction in this study was 11.76%, which suggests that the treatment conditions of *P. simonii* triploid induction remain to be further optimized.

High temperatures can inhibit division in zygotic embryo mitosis and induce zygotic embryo chromosome doubling. In plants, tetraploids can be produced by treating seeds, shoots, somatic embryos, calli and protoplasts with mutagens (Kadota and Niimi 2002; Ewald et al. 2009; Dhooghe et al. 2011). Tetraploid poplars have been produced after inducing chromosome doubling using colchicine on the shoot tips or seeds (Mattila 1961; Ewald et al. 2009). However, asynchronous cell cycles make it difficult to induce chromosome doubling for all cells in multicellular tissue, often resulting in mixoploids. Furthermore, all tetraploids from an explant have the same genotype. In this investigation, chromosome doubling was induced in unicellular zygotic embryos, avoiding mixoploid production. Previous studies have reported that 32 tetraploid poplars (7.41% induction rate) were successfully produced by inducing zygote chromosome doubling with high temperature exposure, and the treatment was guided by observing the developmental characteristics of seed hairs in the ovary in *P. pseudo-simonii* × *P. nigra* 'Zheyin3#' (Wang et al. 2013). Meanwhile, in the suitable seed hair stage III and IV, the hypnozygotes were in the majority, which suggested that the first zygote division was the effective time to apply the mutagenic agent. In this study, 12 tetraploid poplars (5.95% induction rate) were successfully produced and the most suitable stage was guided by the time after pollination, which was more direct. The suitable stage for zygotic embryo chromosome doubling was also the hypnozygote period, suggesting that treating the zygotic embryo during the first division offers

the greatest possibility to produce non-chimeric tetraploids.

Polyploid formations and their applications in genetic breeding

There are two types of mature pollen in plants, bicellular and tricellular types (Ichiro 1993), according to the timing of the generative cell division. For the tricellular type, the division occurs before pollen germination. For the bicellular type, which is the type of poplar mature pollen (Zhang and Yu 2000; Li et al. 1982), division of the generative cell occurs in the pollen tube after germination. In previous research, the generative cell of poplar mature pollen divided at 3–24 HAP (Li et al. 1982; Mireille et al. 1991, 1992; Winton 1968), so treated catkins at 0–36 HAP with colchicine and high temperatures can possibly result in a diploid sperm cell caused by an unreduced division of the generative cell in the pollen tube. However, in our research, the effective period of polyploid production was after 66 HAP, which suggests that the polyploids in this study are not from the fertilization of a diploid sperm cell. As another possibility, high temperatures may inhibit mitosis when a generative cell divides into two sperm cells and produces a diploid ($2n$) sperm cell. Once double fertilization has occurred, it will inevitably result in the production of no embryo or no endosperm, finally leading to embryo abortion or embryo rescue. In our research, all offspring plants were from seed breeding, which suggests that the polyploid formations in this study were not the result of chromosome doubling in the sperm cell. Therefore, in this study, triploids originated from $2n$ female gametes and tetraploids originated from zygotic embryo mitotic inhibition.

There are three modes of $2n$ female gametes formation, including first division restitution (FDR), second division restitution (SDR) of abnormal meiosis during megasporogenesis (Xi et al. 2012), and post-meiotic restitution (PMR) derived from embryo sac chromosome doubling (Wang et al. 2012). Depending on the mechanism for $2n$ gamete formation, the genetic structure in the polyploid progeny varied. Theoretically, the FDR type $2n$ gamete transmits approximately 74.80% parental heterozygosity, and the SDR type can transmit approximately 39.58% to the progeny (Dong et al. 2014). However, different from the former types, completely homozygous $2n$ gametes can arise from the PMR mechanism (Bastiaanssen et al. 1998). In our investigation, all triploid poplars were induced after 66 HAP, while all megasporocytes had completed meiosis at 60 HAP, suggesting that triploids induced in this study originated from the PMR type $2n$ female gametes, which could be presumed to be characterized by complete homozygosity. The significance of homozygous $2n$ female gametes in plant breeding programmes includes the advancement of genetic research and new germplasm production. The triploids in this study provided new germplasm for both cultivar selection and genetic improvement of *Populus*. Originated from zygotic embryo chromosome doubling, tetraploid poplars induced in our investigation have different genotypes and provide important parental germplasms for further triploid breeding.

Polyploidization increases the genetic material of organisms and enhances the production of secondary plant metabolites. The increased genome causes gene dose effects and interaction effects, resulting in changes in resistance, development, and morphological characteristics, such as pest resistance, drought tolerance, flowering time, and organ size. This characteristic gives polyploid species a greater advantage in natural selection and evolution, as they can colonize a wider range of habitats and survive better in harsh, unstable environments compared with their diploid progenitors (Levin 1983; Stebbins 1985; Song et al. 1995; Matzke et al. 1999; Rieseberg et al. 2003). The genetic effects produced by polyploidization provide numerous resources for breeding programmes and a firm foundation for good breeding, which is important for increasing production value. In *Populus*, some triploid cultivars have greater advantages than diploids do, including larger leaves, higher yields, better leaf quality, and greater resistance (Zhu 2006). Furthermore, triploids normally have sterile pollen, thereby precluding pollen pollution and reducing risks of pollen sensitivity in humans.

Synthetic allopolyploids require two nuclear genomes that function normally within a common cytoplasm, which could result in rapid genetic and epigenetic variation, causing changes in gene expression, such as gene silencing and novel expression (Wendel 2000; Osborn et al. 2003; Salmon et al. 2005; Chen 2007). Meanwhile, polyploidization can have an indirect effect on methylation patterns that develop on an evolutionary scale, possibly in response to environmental factors, because genome duplications create opportunities for functional divergence between duplicated genes (Chen 2007; Otto 2007; Li et al. 2011; Aversano et al. 2012). Therefore, the synthetic polyploids produced in this study will be useful for examining genetic and epigenetic changes in poplars (Song et al. 1995; Shaked et al. 2001; Pires et al. 2004; Udall et al. 2005; Chen 2007).

Conclusion

This study is the first to report that triploids and tetraploids have been successfully produced through embryo sac and zygotic embryo chromosome doubling by treating female

catkins with high temperatures in *P. simonii* and its hybrid, *P. simonii* × *P. nigra* var. *Italica*. Furthermore, the relationship between flower bud morphological characteristics (time after pollination) and female meiotic stage (embryo sac and zygotic embryo development) was established to determine the induction treatment period. From this analysis, the best combinations of factors for inducing polyploids were selected. Finally, 37 triploids and 12 tetraploids were detected after treating female catkins with high temperatures which are valuable for genetic research and breeding programmes of *Populus*.

Author contribution statement JZ guided the research. LG, WX and ZW conceived and designed the research. LG, WX and YZ conducted the experiments, including material collection, hybridization, doubling treatment, seedling management, and paraffin sectioning. LG analysed the data. LG and ZW wrote and revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

Aversano R, Capomaccio S, Carputo D, Veronesi F, Rosellini D (2012) Variation of DNA methylation and phenotypic traits following unilateral sexual polyploidization in *Medicago*. Euphytica 186(3):731–739. doi:10.1007/s10681-011-0571-2

Bastiaanssen HJM, Van Den Berg PMMM, Lindhout P, Jacobsen E, Ramanna MS (1998) Postmeiotic restitution in 2*n*-egg formation of diploid potato. Heredity 81(1):20–27. doi:10.1046/j.1365-2540.1998.00370.x

Bradshaw HD Jr, Stettler RF (1993) Molecular genetics of growth and development in *Populus*. I. Triploidy in hybrid poplars. Theor Appl Genet 86(2–3):301–307

Bradshaw HD Jr, Ceulemans R, Davis J, Stettler R (2000) Emerging model systems in plant biology: poplar (*Populus*) as model forest tree. J Plant Growth Regul 19(3):306–313

Cai X, Kang XY (2011) In vitro tetraploid induction from leaf explants of *Populus pseudo-simonii* Kitag. Plant Cell Rep 30(9):1771–1778. doi:10.1007/s00299-011-1085-z

Chen ZJ (2007) Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Annu Rev Plant Biol 58(1):377–406. doi:10.1146/annurev.arplant.58.032806.103835

Dhooghe E, Laere KV, Eeckhaut T, Leus L, Huylenbroeck JV (2011) Mitotic chromosome doubling of plant tissues in vitro. Plant Cell Tissue Organ Culture 104(3):359–373. doi:10.1007/s11240-010-9786-5

Doležel J, Binarová P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31(2):113–120. doi:10.1007/BF02907241

Dong CB, Suo YJ, Wang J, Kang XY (2014) Analysis of transmission of heterozygosity by 2*n* gametes in *Populus* (Salicaceae). Tree Genet Genomes 11(1):1–7. doi:10.1007/s11295-014-0799-9

Ewald D, Ulrich K, Naujoks G, Schröder M-B (2009) Induction of tetraploid poplar and black locust plants using colchicine: chloroplast number as an early marker for selecting polyploids in vitro. Plant Cell Tissue Organ Culture 99(3):353–357. doi:10.1007/s11240-009-9601-3

Harder LD, Thomson JD (1989) Evolutionary options for maximizing pollen dispersal of animal pollinated plants. Am Nat 133(3):323–344

Harder M, Verhagen S, Winton L, Einspahr D (1976) Tetraploid aspen production using unreduced pollen from triploid males. For Sci 22(3):329–330

Ichiro T (1993) Development of male gametes in flowering plants. J Plant Res 106(1):55–63. doi:10.1007/BF02344373

Kadota M, Niimi Y (2002) In vitro induction of tetraploid plants from a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). Plant Cell Rep 21(3):282–286. doi:10.1007/s00299-002-0509-1

Kang XY (1996) Chromosome count and shape of poplar. J Gansu Agric Univ 31(1):67–70

Kang XY, Zhu ZT (1997) A study on the 2*n* pollen vitality and germinant characteristics of white poplars. Acta Bot Yunnanica 19(4):402–406

Kang XY, Zhu ZT, Lin HB (1999) Study on the effective treating period for pollen chromosome doubling of *Populus tomentosa* × *P. bolleana*. Sci Silvae Sin 35(4):21–24

Kang XY, Zhu ZT, Zhang ZY (2000) Breeding of triploids by the reciprocal crossing of *Populus alba* × *P. glandulosa* and *P. tomentosa* × *P. bolleana*. J Beijing For Univ 22(6):8–11

Kang XY, Zhang PD, Gao P, Zhao F (2004) Discovery of a new way of poplar triploids induced with colchicine after pollination. J Beijing For Univ 26(1):1–4

Levin DA (1983) Polyploidy and novelty in flowering plants. Am Nat 122(1):1–25

Li GP, Huang QC (2006) Ontogeny of pollen and pollination in *Keteleeria fortunei*. Sci Silvae Sin 42(5):42–47

Li WD, Fan RW, Mai XL (1982) On the embryological observations of the seed development of *Populus simonii* Carr. Sci Silvae Sin 18(2):113–119

Li YH, Kang XY, Wang SD, Zhang ZH, Chen HW (2008) Triploid induction in *Populus alba* × *P. glandulosa* by chromosome doubling of female gametes. Silvae Genet 57(1):37–40

Li A, Hu BQ, Xue ZY, Chen L, Wang WX, Song WQ, Chen CB, Wang CG (2011) DNA methylation in genomes of several annual herbaceous and woody perennial plants of varying ploidy as detected by MSAP. Plant Mol Biol Rep 29(4):784–793. doi:10.1007/s11105-010-0280-3

Liao T, Cheng SP, Zhu XH, Min Y, Kang XY (2016) Effects of triploid status on growth, photosynthesis, and leaf area in *Populus*. Trees 30(4):1–11. doi:10.1007/s00468-016-1352-2

Lu M, Zhang PD, Kang XY (2013) Induction of 2n female gametes in *Populus adenopoda* Maxim by high temperature exposure during female gametophyte development. Breed Sci 63(1):96–103. doi:10.1270/jsbbs.63.96

Magnard JL, Yang M, Chen YCS, Leary M, McCormick S (2001) The *Arabidopsis* gene tardy asynchronous meiosis is required for the normal pace and synchrony of cell division during male meiosis. Plant Physiol 127(3):1157–1166. doi:10.1104/pp.010473

Mattila RE (1961) On the production of the tetraploid hybrid aspen by colchicine treatment. Hereditas 47(3–4):631–640

Matzke MA, Scheid OM, Matzke AJM (1999) Rapid structural and epigenetic changes in polyploid and aneuploid genomes. BioEssays 21(9):761–767

Mireille R, Najat J, Charlette S, Russell S, Dumas C (1991) Male gametophyte development and formation of the male germ unit in *Populus deltoides* following compatible pollination. Protoplasma 162(2):140–150. doi:10.1007/BF02562557

Mireille R, Najat J, Charlette S, Russell S, Dumas C (1992) Interspecific incompatibility in *Populus*: inhibition of tube growth and behaviour of the male germ unit in *P. deltoides* × *P. alba* cross. Protoplasma 168(3):107–110. doi:10.1007/BF01666256

Mofidabadi AJ, Modir-Rahmati AR, Tavesoli A (1998) Application of ovary and ovule culture in *Populus alba* L. × *P. euphratica* OLIV. hybridization. Silvae Genet 47(5):332–334

Müntzing A (1936) The chromosomes of a giant *Populus tremula*. Hereditas 21(2–3):383–393

Nilsson-Ehle H (1936) Note regarding the gigas from of *Populus tremula* found in nature. Hereditas 21:372–382

Nomura K, Nakajima J, Ohta H, Kagawa H, Tanaka H, Unuma T, Yamauchi K, Arai K (2004) Induction of triploidy by heat shock in the Japanese eel *Anguilla japonica*. Fish Sci 70(2):247–255. doi:10.1111/j.1444-2906.2003.00798.x

Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee HS, Comai L, Madlung A, Doerge RW, Colot V, Martienssen RA (2003) Understanding mechanisms of novel gene expression in polyploids. Trends Genet 19(3):141–147. doi:10.1016/S0168-9525(03)00015-5

Otto SP (2007) The evolutionary consequences of polyploidy. Cell 131(3):452–462. doi:10.1016/j.cell.2007.10.022

Pires JC, Zhao JW, Schranz ME, Leon EJ, Quijada PA, Lukens LN, Osborn TC (2004) Flowering time divergence and genomic rearrangements in resynthesized *Brassica* polyploids (Brassicaceae). Biol J Linn Soc 82(4):675–688. doi:10.1111/j.1095-8312.2004.00350.x

Randolph LF (1932) Some effects of high temperature on polyploidy and other variations in maize. Proc Natl Acad Sci USA 18(3):222–229

Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Lexer C (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. Science 301(5637):1211–1216. doi:10.1126/science.1086949

Risso-Pascotto C, Pagliarini MS, Do Valle CB, Jank L (2004) Asynchronous meiosis in an interspecific hybrid of *Brachiaria ruziziensis* and *B. brizantha*. Plant Cell Rep 23(5):304–310. doi:10.1007/s00299-004-0802-2

Robinson JP (2006) DNA content measurement for DNA ploidy and cell cycle analysis. In: Darzynkiewicz Z, Juan G (eds) Current protocols in cytometry. Wiley, New York, pp 1–24

Salmon A, Ainouche ML, Wendel JF (2005) Genetic and epigenetic consequences of recent hybridization and polyploidy in *Spartina* (Poaceae). Mol Ecol 14(4):1163–1175. doi:10.1111/j.1365-294X.2005.02488.x

Shaked H, Kashkush K, Ozakan H, Feldman M, Levy AA (2001) Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome to wide hybridization and allopolyploidy in wheat. Plant Cell 13(8):1749–1759. doi:10.1105/TPC.010083

Song K, Lu P, Tang K, Osborn TC (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploidy evolution. Proc Natl Acad Sci USA 92(17):7719–7723. doi:10.1073/pnas.92.17.7719

Stebbins GL (1985) Polyploidy, hybridization and invasion of new habitats. Ann Mo Bot Gard 72(4):824–832. doi:10.2307/2399224

Tavoletti S (1994) Cytological mechanisms of 2n egg formation in a diploid genotype of *Medicago sativa* subsp. *falcate*. Euphytica 75(1–2):1–8. doi:10.1007/BF00024525

Udall JA, Quijada PA, Osborn TC (2005) Detection of chromosomal rearrangements derived from homeologous recombination in four mapping populations of *Brassica napus* L. Genetics 169(2):967–979. doi:10.1534/genetics.104.033209

Wang J, Kang XY, Li DL, Jing YC, Zhang ZH (2006) Meiosis and chromosome behavior of pollen mother cell in *Populus simonii* Carr. × *P. nigra* L. 'Tongliao'. Acta Bot Boreali Occident Sin 26(11):2231–2238

Wang J, Kang XY, Li DL (2008) Chromocenter number and relationship between it and ploidy level in *Populus*. Acta Bot Boreali Occident Sin 28(10):1968–1971

Wang J, Kang XY, Li DL, Chen HW, Zhang PD (2010) Induction of diploid eggs with colchicine during embryo sac development in *Populus*. Silvae Genet 59(1):40–48

Wang J, Kang XY, Li DL (2012) High temperature-induced triploid production during embryo sac development in *Populus*. Silvae Genet 61(3):85–93

Wang J, Shi L, Song SY, Tian J, Kang XY (2013) Tetraploid production through zygotic chromosome doubling in *Populus*. Silva Fenn 47(2):1–12

Wei ZZ, Du QZ, Zhang JF, Li BB, Zhang DQ (2013) Genetic diversity and population structure in Chinese indigenous poplar (*Populus simonii*) populations using microsatellite markers. Plant Mol Biol Rep 31(3):620–632. doi:10.1007/s11105-012-0527-2

Wendel JF (2000) Genome evolution in polyploids. Plant Mol Biol 42(l):225–249. doi:10.1007/978-94-011-4221-2_12

Winton LL (1968) Fertilization in forced quaking aspen and cottonwood. Silvae Genet 17(1):20–21

Winton LL, Einspahr DW (1970) Tetraploid aspen production using unreduced triploid pollen. For Sci 16(2):180–182

Wyatt R (1982) Inflorescence architecture: how flower number, arrangement, and phenology affect pollination and fruit set. Am J Bot 69(4):585–594

Xi XJ, Li D, Xu WT, Guo LQ, Zhang JF, Li BL (2012) 2*n* egg formation in *Populus* × *euramericana* (Dode) Guinier. Tree Genet Genomes 8(6):1237–1245. doi:10.1007/s11295-012-0510-y

Xi XJ, Guo LQ, Xu WT, Zhang JF, Li BL (2014) Megasporogenesis, megagametogenesis and induction of 2*n* eggs with colchicine in section Aigeiros. Scand J For Res 29(6):527–536. doi:10.1080/02827581.935472

Xu JR, Li YY (2014) Experiments design in forestry. China Agricultural Press, Beijing

Yang H, Guo X (2006) Polyploid induction by heat shock-induced meiosis and mitosis inhibition in the dwarf surfclam, *Mulinia lateralis* Say. Aquaculture 252(2):171–182. doi:10.1016/j.aquaculture.2005.07.017

Yu JL, Wang HJ, Hou JM, Zhang J, Qiao GJ, Jin ZH (2008) Investigation of flexural strength and porosity of porous Si3N4 ceramics through orthogonal experimental design. J Ceram Process Res 9(6):624–629

Zhang ZY, Yu XS (2000) Advances in reproduction biology of poplars. J Beijing For Univ 22(6):69–74

Zhang S, Qi L, Chen C, Li X, Song W, Chen R, Han S (2004) A report of triploid *Populus* of the section *Aigeiros*. Silvae Genet 53(2):69–75

Zhang JF, Wei ZZ, Li D, Li BL (2009) Using SSR markers to study the mechanism of 2*n* pollen formation in

Populus × *euramericana* (Dode) Guinier and *P.* × *popularis*. Ann For Sci 66(5):53–62. doi:10.1051/forest/2009032

Zhu ZT (2006) Genetic improvement of *Populus tomentosa*. China Forestry Press, Beijing

Zhu ZT, Lin HB, Kang XY (1995) Studies on allotriploid breeding of *Populus tomentosa* B301 clones. Sci Silvae Sin 31(6):499–505

Zhu ZT, Kang XY, Zhang ZY (1998) Studies on selection of natural triploid of *Populus tomentosa*. Sci Silvae Sin 34(4):22–31