

# *In Vitro* Amphidiploid Induction of A Distant Hybrid *Populus Simonii* × *P. Euphratica* Cv. 'Xiaohuyang-2' and its Effect on Plant Morphology and Anatomy

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## Research Article

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# Abstract

In plants, highly gametic sterility of distant hybrids usually restricts their utilization in breeding programs. Amphidiploid induction produced by somatic chromosome doubling of distant hybrids can effectively restore their gametic fertility. In this study, nodal-segment and leaf explants of a distant hybrid *Populus simonii* × *P. euphratica* cv. 'Xiaohuyang-2' were used to induce chromosome doubling with colchicine *in vitro*. Although chromosome doubling of the nodal-segment explants only produced mixoploids, the treatments of leaf explants on adventitious bud regeneration medium successfully produced 4 amphidiploids, which might be attributed to the direct organogenesis of the adventitious buds on the leaf explants. The highest amphidiploid induction frequency was 16.7%. Both the explant survival rate and polyploidization frequency were significantly affected by colchicine concentration and exposure time. The amphidiploid plants were significantly differed from the diploid and mixoploid plants on morphological and anatomical characteristics. They had larger, thicker, and greener leaves than the diploids and mixoploids. The changes of stomatal features also accompanied with increase of ploidy level. The induced amphidiploid plants of the distant hybrid 'Xiaohuyang-2' are expected to play important roles in breeding programs of *Populus* in future, which can be used as a bridge parent with ability of unreduced gamete formation to cross with fast-growth germplasms to produce triploids pyramiding desirable traits of fast growth, easy cutting propagation, and salt and drought tolerances.

# Introduction

Distant hybridization is an important approach for promoting germplasm introgression and creation of new varieties (Kumar et al. 2011; Girke et al. 2012). However, distant hybrids are usually highly reproductive sterile, owing to failure of meiotic synapsis, imbalanced chromosome segregation and chromosomal elimination (Trojak-Goluch and Berbeć 2003). The highly gametic sterility restricts the employment of distant hybrids in subsequent breeding programs. In order to integrate the distant hybrids to the breeding programs as bridge parents for gene introgression or distant chromosome substitution, it is an alternative way to modify the distant hybrids to amphidiploids to restore their gametic fertility (Kumar et al. 2011). In many crops, such as *Cucumis*, *Triticum*, and *Brassica*, amphidiploids have been played important roles in breeding programs (Chen et al. 2003; Chen and Wu 2008; Li et al. 2021).

Recently, a distant hybrid 'Xiaohuyang-2', derived from intersectional cross between *Populus simonii* and *P. euphratica*, passed the variety examination and approval of Inner Mongolia Autonomous Region, P. R. China. It is a male variety, with parental merits, such as good rooting capability of cuttings, and excellent drought and salinity tolerance (Tian et al. 2016; Qi et al. 2020). However, compared with the species of *Populus* section Aigeiros, the variety still grows slowly. It is necessary to further improve the variety 'Xiaohuyang-2'. Wang et al. (2015) found that the microsporocyte meiosis of 'Xiaohuyang-2' is highly abnormal in chromosome pairing and appointment, causing sterile of gametes. It is necessary to induce amphidiploid induction from the distant hybrid 'Xiaohuyang-2'.

Induction of amphidiploids from distant hybrids commonly relies on somatic chromosome doubling. In the previous studies, both leaves and nodal-segments were used as explants for chromosome doubling of *Populus* (Ewald et al. 2009; Cai et al. 2011; Liu et al. 2018; Zeng et al. 2019). Cai et al. (2011) screened suitable medium of adventitious bud induction from leaf explant of *P. pseudo-simonii*, and obtained 36 tetraploids by treating the adventitious buds with colchicine solution. Zeng et al. (2019) induced 6 hexaploids after oryzalin treatment to nodal sections of a triploid hybrid (*P. alba* × *P. glandulosa*) × *P. tomentosa*. In *Populus*, however, no case is reported on the amphidiploid induction.

In a previous study, *in vitro* regeneration system of the distant hybrid 'Xiaohuyang-2' has been established (Cui et al. 2021). In our study, in order to produce amphidiploid for further breeding utilization, techniques of somatic chromosome doubling of 'Xiaohuyang-2' were investigated from nodal-segment and leaf explants based on the *in vitro* regeneration system. Moreover, the morphological and anatomical variations were analyzed among diploid and induced mixoploid and amphidiploid cytotypes to reveal the influences of polyploidization.

## Materials And Methods

### Plant materials

One-year-old branches of *P. simonii* × *P. euphratica* cv. 'Xiaohuyang-2' were collected from Dengkou County of Inner Mongolia Autonomous Region, P. R. China. The branches were cultured in a greenhouse (15–25°C) to enforce bud sprouting and growth. According to Cui et al. (2021), the rooting aseptic plantlets were obtained from the fresh stem explants. The nodal-segments and leaves were collected from the aseptic plantlets as explants for somatic chromosome doubling.

### Colchicine treatment of chromosome doubling

For treatments of nodal-segments, the 2 cm segments were immersed into liquid MS medium containing 0.05%, 0.10% and 0.20% colchicine for 24, 48 and 72 h in darkness, respectively. Subsequently, the treated nodal-segments were washed with sterile distilled water for three times and transferred to fresh solid rooting medium ( $\frac{1}{2}$ MS medium containing 0.1 mg L<sup>-1</sup> IBA, 30 g L<sup>-1</sup> sucrose, and 6 g L<sup>-1</sup> agar, pH 5.8–6.0). All treatments were replicated for three times and ten segments were used in each treatment. After 50 d culture in a growth room with an illumination of 30–40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 16 h photoperiod at 25°C, the number of survived nodal-segments in each treatment was recorded to calculate survival rate.

For treatments of leaf explants, fully expanded leaves were trimmed into 1 cm × 1 cm pieces crossing the main vein. The leaf pieces were cultured on adventitious bud regeneration medium (MS medium containing 0.4 mg L<sup>-1</sup> 6-BA, 0.4 mg L<sup>-1</sup> KT, 30 g L<sup>-1</sup> sucrose, and 6 g L<sup>-1</sup> agar, pH 5.8–6.0) with the adaxial side touching the medium for 10 days and then transferred to liquid MS medium containing 0.01%, 0.02% and 0.03% colchicine for 24, 48 and 72 h in darkness. After colchicine treatment, the leaf pieces were rinsed in sterile distilled water 5 times and dried with sterile filter paper. Then, the leaf pieces were placed on the fresh adventitious bud regeneration medium without colchicine for differentiation.

The experiments were repeated three times with thirteen explants per treatment. After 50 d culture, the number of survived leaf explants in each treatment was recorded and the regenerated shoots over 1 cm were excised and placed on the fresh solid rooting medium for root induction.

## Detection of ploidy levels

The ploidy levels of the regenerated plantlets were first detected by flow cytometry according to the method of Wang et al. (2012). For the flow cytometric analysis, three or four young leaves of each plantlet were randomly selected and mixed to chop in modified Galbraith's buffer (45 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mM MOPS, 30 mM sodium citrate, 0.5% Triton X-100, and 1% PVP-10, pH 7.0; Galbraith et al. 1983) on ice using a sharp razor blade to distinguish mixoploids. The nuclear suspension was filtered through a 40  $\mu\text{m}$  nylon mesh to remove large debris and stained with 5  $\mu\text{g mL}^{-1}$  DAPI to analyze with a CyFlow Ploidy Analyzer (Partec). The variety 'Xiaohuyang-2' was used as the diploid criteria.

The putative amphidiploid plantlets were further confirmed by somatic chromosome counting according to Wang et al. (2017). Root tips were collected from the plantlets and pretreated with a saturated solution of paradichlorobenzene for 4 h at 25°C. Subsequently, the materials were fixed in fresh Carnoy's solution (ethanol/acetic acid, 3:1) for 24 h at 4°C, and then digested with an enzyme mixture containing 2% cellulose and 2% pectinase dissolved in 0.01 M citrate buffer (pH 4.5) at 37°C for 1.5 h. After carefully washing in distilled water, the softened materials were transferred into fresh Carnoy's solution and prepared cell suspension. The cell suspension was dropped onto a microscope slide and air-dried to prepare the chromosome preparations. After staining with Giemsa solution, the chromosome preparations were observed using an Olympus BX51 microscope.

## Morphological and anatomical investigation

The fourth fully expanded leaves of diploid, mixoploid ( $2x:4x = \sim 1:1$ ) and amphidiploid plantlets after 30 day-rooting culture were collected to compare the morphological and anatomical changes. Ten repetitions were done. The leaf length and width were measured, and the number of leaf serrations was recorded. Then, leaves were fixed in FAA fixative (70% ethanol/acetic acid/40% formaldehyde, 90:5:5) and were subsequently prepared to 8  $\mu\text{m}$ -thick paraffin sections according to standard protocol to measure the leaf thickness. For stomatal feature measurement, the lower epidermis of the leaves was smeared with clear nail polish and placed on a microscope slide. The preparations were then observed under an Olympus BX51 microscope. The stomatal lengths and widths of 20 randomly selected stomata from each preparation were measured. Then, 15 microscopic field areas were randomly selected per leaf to record stomatal density.

## Data statistics

Statistical analyses were performed using SPSS v. 22.0 statistical software. The results presented in this study are mean  $\pm$  SE. Before performing analysis of variance (ANOVA), percentage data were transformed to the arcsin of the square root of  $p/100$ . When treatments differed significantly, Duncan's multiple range

test was used for pairwise comparison. Differences were determined to be statistically significant at  $P < 0.05$ , and highly significant at  $P < 0.01$ .

## Results

### Chromosome doubling of nodal-segment explants

The survival rate of the nodal-segment explants was highly influenced by both colchicine concentration and exposure time ( $P = 0.0008$  and  $P < 0.0001$ , respectively), but not significant by their interaction ( $P = 0.9811$ ). The low colchicine concentration and short exposure time were benefit for the survival of nodal-segment explants (Table 1).

Table 1  
Colchicine induced chromosome doubling of nodal-segment explants.

Treatment	Colchicine concentration (%)	Exposure time (h)	Survival rate (%)	Mixoploid frequency (%)
N1	0.05	24	97.78 ± 12.24 a	0 d
N2	0.05	48	84.44 ± 4.04 bcd	0 d
N3	0.05	72	77.78 ± 9.29 cd	14.07 ± 2.06 c
N4	0.10	24	93.33 ± 15.49 ab	0 d
N5	0.10	48	80.00 ± 6.51 cd	16.82 ± 0.82 bc
N6	0.10	72	68.89 ± 5.99 de	25.89 ± 1.74 b
N7	0.20	24	86.67 ± 8.00 bc	21.25 ± 6.37 bc
N8	0.20	48	66.67 ± 5.19 de	37.21 ± 5.09 a
N9	0.20	72	55.56 ± 5.27 e	45.73 ± 7.23 a

Note: Lower-case letters indicate significance differences among the treatments based on Duncan's test at  $P < 0.05$ .

Chromosome doubling of the nodal-segment explants did not produce amphidiploid but 49 mixoploids (Table 1). The mixoploid induction frequency was significantly affected by colchicine concentration, exposure time and their interaction ( $P < 0.0001$ ,  $P < 0.0001$ , and  $P = 0.0002$ , respectively). In the N9

treatment (0.20% colchicine and 72 hours exposure), the induction frequency of mixoploids was the highest at 45.73%±7.23, although the survival rate was the lowest in this treatment.

## Chromosome doubling of leaf explants

Based on ANOVA, the survival rate of leaf explants was significantly influenced by colchicine concentration, exposure time and their interaction ( $P < 0.0001$ ). In the L1 treatment (0.01% colchicine and 24 hours exposure), the survival rate of explants was the highest (76.28% ± 0.64). However, in the L9 treatment (0.03% colchicine and 72 hours exposure), all explants died after treatment.

Four amphidiploids were detected from the regenerated adventitious buds of treated leaf explants (Table 2, Figure 1). The highest amphidiploid induction frequency was 16.67% at the L5 treatment (0.02% colchicine and 48 hours exposure). The amphidiploid induction frequencies of the treatment L3 (0.01% colchicine and 72 hours exposure) and L6 (0.02% colchicine and 72 hours exposure) were 11.11% and 6.67%, respectively.

Table 2  
Colchicine induced chromosome doubling of leaf explants.

Treatment	Colchicine concentration (%)	Exposure time (h)	Survival rate (%)	Number of amphidiploids	Amphidiploid induction frequency (%)
L1	0.01	24	76.28 ± 0.64 A	0	0
L2	0.01	48	57.51 ± 2.23 B	0	0
L3	0.01	72	38.46 ± 0 CD	1	11.11
L4	0.02	24	51.11 ± 1.11 B	0	0
L5	0.02	48	41.67 ± 0 C	2	16.67
L6	0.02	72	30.51 ± 0.26 D	1	6.67
L7	0.03	24	34.34 ± 1.01 CD	0	0
L8	0.03	48	18.61 ± 4.20 E	0	0
L9	0.03	72	0 F	0	0

Note: Upper-case letters indicate significance differences among the treatments based on Duncan's test at  $P < 0.01$ .

# Ploidy-related variation in leaf morphology and anatomy

The change of ploidy levels in 'Xiaohuyang-2' resulted in morphological and anatomical variations of leaves (Table 3). In visual, the amphidiploid plants had larger, thicker, and greener leaves than the diploid and mixoploid plants (Figure 2). After measurement, the average leaf length of the amphidiploids ( $3.83 \pm 0.10$  cm) was significantly longer than that of the diploids ( $3.42 \pm 0.12$  cm) and mixoploids ( $3.48 \pm 0.12$  cm) ( $P = 0.0442$ ). The average leaf width of both the amphidiploids ( $1.07 \pm 0.04$  cm) and mixoploids ( $0.98 \pm 0.03$  cm) were highly significantly wider than that of the diploids ( $0.71 \pm 0.03$  cm) ( $P < 0.0001$ ). The leaf length/width ratio of diploids ( $4.82 \pm 0.16$ ) was highly significantly bigger than that of the amphidiploids ( $3.61 \pm 0.09$ ) and mixoploids ( $3.56 \pm 0.14$ ) ( $P < 0.0001$ ). The serrations on leaf margin in amphidiploids ( $13.14 \pm 0.59$ ) was also highly significantly more than that in diploids ( $9.29 \pm 0.97$ ) and mixoploids ( $9.57 \pm 0.81$ ) ( $P = 0.0053$ ). Observation of paraffin sections of leaves showed that the leaf thickness of amphidiploids was statistically same with that of mixoploids, and both were significantly thicker than that of the diploids ( $P = 0.0093$ ).

Table 3  
Comparison analysis of leaf and stomatal traits of plantlets with different ploidy levels.

Traits	Diploid	Mixoploid	Amphidiploid
Leaf length (cm)	$3.42 \pm 0.12$ a	$3.48 \pm 0.12$ a	$3.83 \pm 0.10$ a
Leaf width (cm)	$0.71 \pm 0.03$ B	$0.98 \pm 0.03$ A	$1.07 \pm 0.04$ A
Leaf length/width ratio	$4.82 \pm 0.16$ A	$3.56 \pm 0.14$ B	$3.61 \pm 0.09$ B
Serration number on leaf margin	$9.29 \pm 0.97$ B	$9.57 \pm 0.81$ B	$13.14 \pm 0.59$ A
Leaf thickness ( $\mu\text{m}$ )	$186.77 \pm 4.43$ B	$264.57 \pm 21.81$ A	$257.74 \pm 13.42$ A
Stomatal length ( $\mu\text{m}$ )	$22.74 \pm 1.04$ B	$23.08 \pm 0.69$ B	$31.68 \pm 0.83$ A
Stomatal width ( $\mu\text{m}$ )	$13.02 \pm 0.45$ B	$15.06 \pm 0.71$ AB	$16.60 \pm 0.56$ A
Stomatal density ( $\text{n mm}^{-2}$ )	$100.1 \pm 3.6$ a	$93.5 \pm 15.7$ a	$57.6 \pm 5.2$ b

Note: Lower-case and upper-case letters indicate significance differences among the ploidy levels based on Duncan's test at  $P < 0.05$  and  $P < 0.01$  respectively.

## Changes in stomatal characteristics

Variations of stomatal length, width and density were analyzed among the diploids, mixoploids and amphidiploids. In average, the stomatal length of amphidiploids was  $31.68 \pm 0.83$   $\mu\text{m}$ , which was highly significantly larger than that of both diploids ( $22.74 \pm 1.04$   $\mu\text{m}$ ) and mixoploids ( $23.08 \pm 0.69$   $\mu\text{m}$ ). The stomatal width of amphidiploids ( $16.60 \pm 0.56$   $\mu\text{m}$ ) was also highly significantly larger than that of the diploids ( $13.02 \pm 0.45$   $\mu\text{m}$ ). The stomatal density of amphidiploids ( $57.61 \pm 5.21$   $\text{n mm}^{-2}$ ) was significantly lower than that of both diploids ( $100.11 \pm 3.63$   $\text{n mm}^{-2}$ ) and mixoploids ( $93.50 \pm 15.67$   $\text{n mm}^{-2}$ ).

mm<sup>-2</sup>). In aspect of the frequency distribution of the stomatal characteristics, the Figure 3 shows that the frequencies of stomatal lengths and widths follow approximate Gaussian distributions in both diploid and amphidiploid. In contrast, the frequencies of both stomatal lengths and widths of the mixoploid were bimodal distributions.

## Discussion

Although nodal-segments were successfully used to induce somatic chromosome doubling of plants (Zeng et al. 2019), only mixoploids were produced after colchicine treatment of the nodal-segments of 'Xiaohuyang-2' in the present study. Mixoploids are considered as fewer valuable materials than homogeneous polyploid plants due to their defect in genetic stability (Dhooghe et al. 2011; Rose et al. 2000). In breeding program, it is necessary to isolate homogeneous polyploids from the mixoploids. Liu et al. (2020) developed a method for isolation of diploid and tetraploid cytotypes from mixoploids based on adventitious bud regeneration in *Populus*. This method could be used to isolate the amphidiploid cytotype of 'Xiaohuyang-2' from the mixoploids in future.

In the previous studies, leaf explants were effectively used to induce somatic chromosome doubling of *Populus* in vitro based on adventitious bud regeneration (Cai and Kang 2011; Xu et al. 2018; Liu et al. 2018). In our study, 4 amphidiploids were induced based on the colchicine treatments of leaf explants of 'Xiaohuyang-2'. Cui et al. (2021) found that the adventitious buds of the 'Xiaohuyang-2' were regenerated directly from the incisions of leaf explants, without callus formation. The direct organogenesis of adventitious buds was considered as single cell origin (Broertjes and van Harten 1985), which could be benefit for the homogeneous amphidiploid/tetraploid induction in our study. However, the number of amphidiploids and the frequency of induction were low. According to Cui et al. (2021), the induction of adventitious buds of 'Xiaohuyang-2' is difficult, only 1.80 ( $\pm$  0.66) induced buds per leaf explant, which is probably the reason of low amphidiploid induction frequency in our study.

Formation of amphidiploids usually result in complex phenotypic variation (Choudhary et al. 2000; Chen and Wu 2008). In our study, compared to diploid 'Xiaohuyang-2', the amphidiploids exhibited significantly thicker leaves, larger mesophyll cells, more serrations on leaf margin, larger stomata, and lower stomatal density, and the morphological and anatomic characteristics of mixoploids were intermediate between the diploids and amphidiploids, indicating that phenotypic variation was highly related with change of ploidy level. Polyploids often thrive in harsh or disturbed environments, with strong capability to response both biotic or abiotic stress (Van de Peer et al. 2021). Kiani et al. (2021) found that the amphidiploids of hybridization between wheat and *Aegilops cylindrica* exhibited significantly higher tolerance to salt stress. Although the diploid distant hybrid 'Xiaohuyang-2' have already possess good rooting capability of cuttings and excellent drought and salinity tolerance, the amphidiploids may have increase tolerance to abiotic stresses, which could need to be further investigated.

To overcome gametic sterility, distant hybrids are usually induced into amphidiploids in crops, which can function as a bridge parent for gene introgression or chromosome substitution (Kumar et al. 2011; Liu et



al. 2014). In the present study, amphidiploids of a distant hybrid *P. simonii* × *P. euphratica* cv. 'Xiaohuyang-2' were induced by somatic chromosome doubling. Different from the highly sterility of distant hybrids, amphidiploids are theoretically characterized by regular meiotic division, resulting in viable 2n gamete production. In *Populus* breeding program, 2n gamete induction have played a dominant role in triploid production (Kang et al. 2000; Wang et al. 2010, 2012; Li et al. 2019), which produced many varieties with good performance in growth, adaptability, and pulpwood properties (Kang 2016). The production of amphidiploids in the present study will satisfy the demand of 2n gametes for triploid breeding of *Populus*. In our program, fast growing species in *Populus* section Aigeiros could cross with the amphidiploid to produce triploid progeny characterized by fast growth, good rooting capability of cuttings, and excellent drought and salinity tolerance.

## Conclusion

In this study, we first induced amphidiploids of a distant hybrid *Populus simonii* × *P. euphratica* cv. 'Xiaohuyang-2' by *in vitro* somatic chromosome doubling with colchicine. The results showed that the treatments of leaf explants on adventitious bud regeneration medium successfully produced 4 amphidiploids, but chromosome doubling of the nodal-segment explants only produced mixoploids, which might be attributed to the direct organogenesis of the adventitious buds on leaf explants. The highest amphidiploid induction frequency was 16.7% at treatment of 0.02% colchicine and 48 hours exposure with 10 days of preculture. Both the explant survival rate and polyploidization frequency were significantly affected by colchicine concentration and exposure time. The amphidiploids exhibited significantly thicker leaves, larger mesophyll cells, more serrations on leaf margin, larger stomata, and lower stomatal density than that of the diploids, and the morphological and anatomic characteristics of mixoploids were intermediate between the diploids and amphidiploids, indicating that phenotypic variation was highly related with change of ploidy level. The induced amphidiploids were expected with restored gametic fertility to play important roles in breeding programs of *Populus*.

## Declarations

### Author contribution statement

JW conceived and designed research. XXZ, YZ, and XTC conducted chromosome doubling experiments and analyzed data. DLL and HYZ conducted the morphological and anatomical analysis. WD provided the plant material. XXZ and JW wrote the manuscript. All authors read and approved the manuscript.

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### Conflict of interest

The authors declare that they have no conflict of interests.

## Data archive statement

All data generated or analyzed during this study are included in this article. The data are available from the corresponding author on reasonable request.

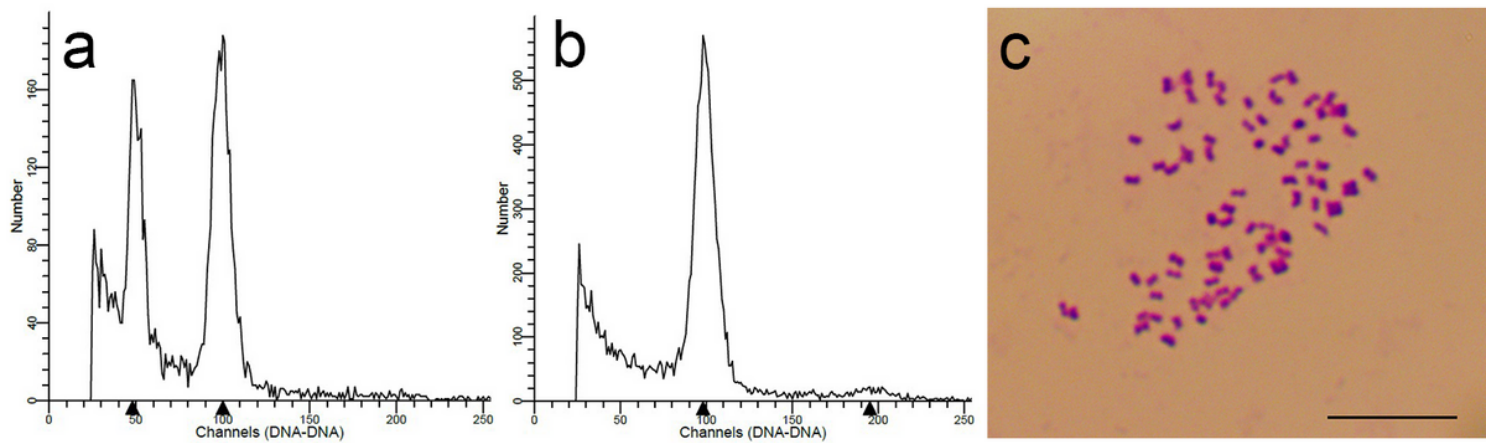
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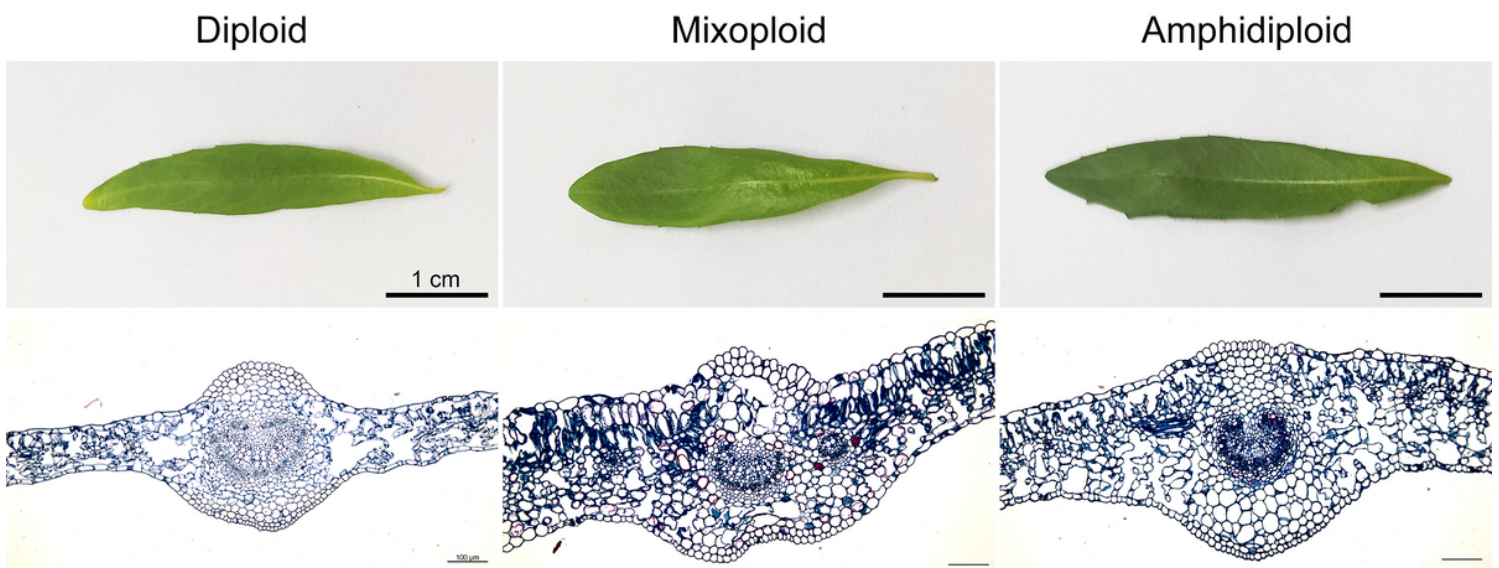
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## Figures



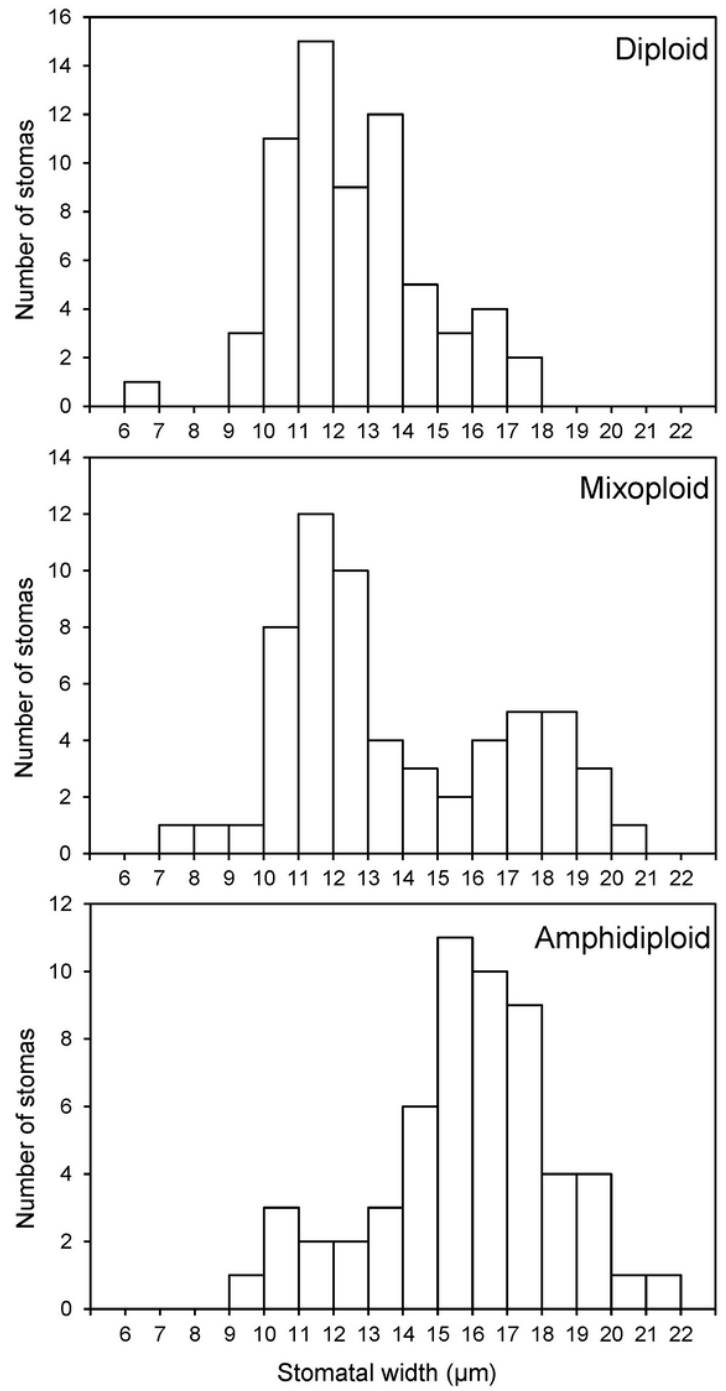
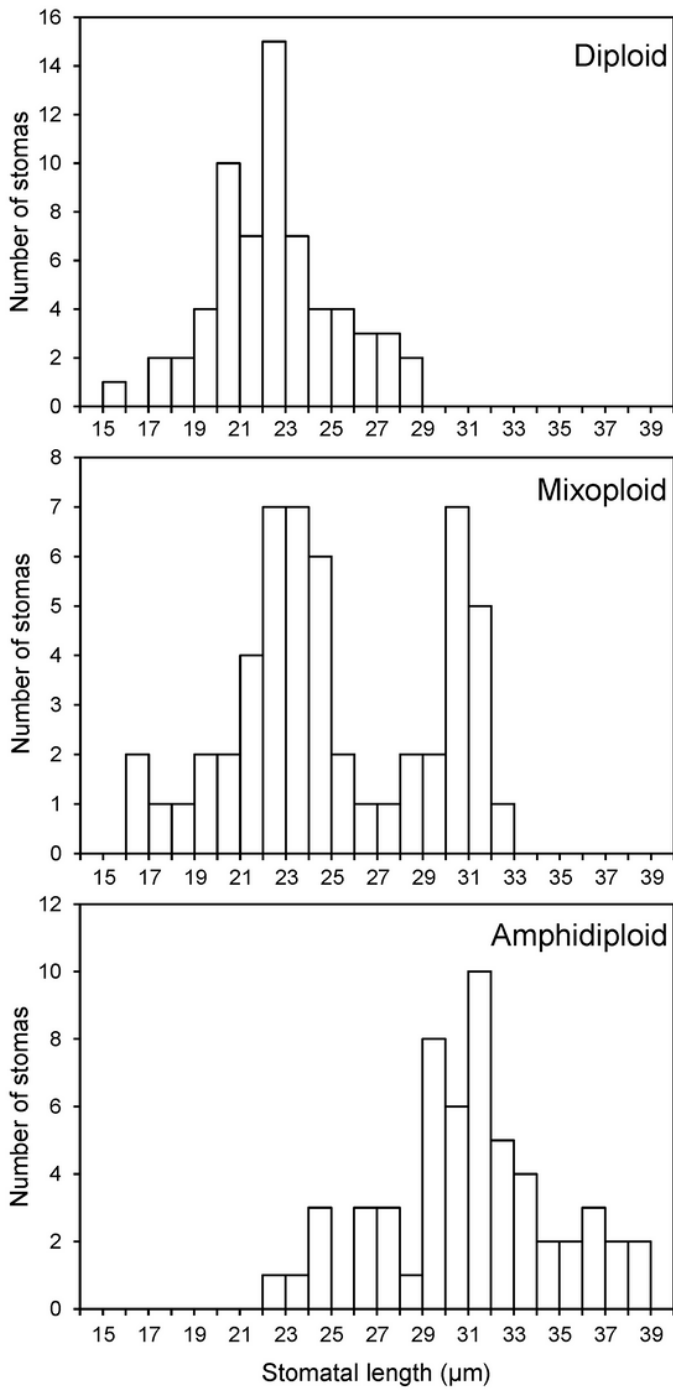
**Figure 1**

Detection of ploidy levels. (a) Flow cytometric analysis of a mixoploid; (b) Flow cytometric analysis of an amphidiploid; (c) Somatic chromosome counting of the amphidiploid.



**Figure 2**

Leaf morphological and anatomical characteristics of diploid, mixoploid and amphidiploid.



**Figure 3**

Frequency distribution histograms of stomatal length and width of different ploidy tissue culture plantlets of 'Xiaohuyang-2'.