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ORIGINAL ARTICLE

# Autopolyploid induction via somatic embryogenesis in *Lilium distichum* Nakai and *Lilium cernuum* Komar

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## Abstract

New ornamental varieties of high quality can be created via artificial polyploid induction. In the present study, the first system of polyploid induction with somatic embryogenesis of *Lilium distichum* Nakai and *Lilium cernuum* Komar. was developed. Somatic embryos were cultured on MS with 0.41 μmol $L^{-1}$ picloram and 1.07 μmol $L^{-1}$ NAA by scales (5 $mm^2$). After 40 days, somatic embryos were transferred to MS with 2.21 μmol $L^{-1}$ BA for somatic embryogenesis. As determined from observations of paraffin sections, embryonic cells of *L. distichum* originated from outer cells at first, and somatic embryogenesis occurred through an indirect pathway. In *L. cernuum*, embryonic cells originated from inner cells at first, and somatic embryogenesis occurred through a direct pathway. Polyploids were successfully formed from somatic embryos and scales by the soaking and mixed culture methods with different colchicine concentrations (0.01%, 0.05%, and 0.1%; v/v) and durations (24, 48, and 72 h). The polyploid induction rate reached 57.14% and 46.15% with 0.05% colchicine treatment in *L. distichum* (48 h) and *L. cernuum* (24 h), respectively. Tetraploids (28.57% and 23.08%) and aneuploids without chimeras among the obtained polyploid plantlets were identified by chromosome counts of root-tip tissue squashes in *L. distichum* and *L. cernuum*. Tetraploid plantlets of *L. distichum* exhibited broader leaves, longer guard cells, larger stomata and higher stomatal conductance than diploid plantlets. Tetraploid plantlets of *L. cernuum* showed 1.76 × higher chlorophyll content, significantly more leaves, longer guard cells, larger stomata and lower stomatal conductance than diploid plantlets.

## Key message

For the first time, we established a somatic embryogenesis system for *L. distichum* and *L. cernuum* using somatic embryos and scales to induce polyploids by soaking and mixed culture. Combinations of colchicine concentrations and periods of time were compared to select the best treatment combination. Comprehensive morphological observations, stomatal observations and root-tip tissue squashes were used to identify the ploidy of doubling plants. Our results provide a foundation for improving the ornamental value of two wild lily species, creating new Lilium germplasm and improving the reproduction coefficient of these resources.

**Keywords** *Lilium distichum* Nakai · *Lilium cernuum* Komar · Somatic embryogenesis · Autopolyploid induction

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## Abbreviations

| | |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| BA | 6-Benzyladenine |
| NAA | Naphthylacetic acid |
| MS | Murashige and Skoog medium |
| Picloram | 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid |

## Introduction

Lily (*Lilium* spp.) cultivation has a long history with a "Bullbar flower king" reputation. It has high value in concept, medicine and commerce. According to statistics,

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approximately 55 of the 115 species of Liliaceae originated in China (Chen et al. 2011). Rich wild resources are valuable treasures in the world flower germplasm resources bank, and also provide favorable conditions for breeding work. *Lilium distichum* Nakai (*L. distichum*) and *Lilium cernuum* Komar. (*L. cernuum*) are diploid species in the family Liliaceae and are native to northeast China. *L. distichum* is an economically important ornamental plant that is valued for its flowers, which present a rolled perianth and pale orange color, and its wheeling leaves located in the middle of the stalks. However, this species has a low flowering rate and is susceptible to disease in summer (Lei and Pan 2009). *L. cernuum* is a rare and endangered wild resource with a rolled perianth and rare lavender petals. Unfortunately, it is easily damaged, has a long breeding cycle and is prone to lodging in the rainy season (Zhou 2006). These shortcomings limit the application of these two resources. Therefore, it is very urgent and important to improve these two lily species to obtain new germplasms with excellent ornamental traits.

Polyploidization is an important method of germplasm improvement, and approximately 70% of newly produced angiosperms in nature are polyploids (Otto and Whitton 2000; Otto 2007) representing sources of heredity, biochemistry and evolution (Soltis and Soltis 2016). Genome doubling result in new phenotypes, some with high visibility to natural selection, such as organ size and flowering time (Adams and Wendel 2005). Therefore, the phenomena caused by polyploidy have gradually become a research hotspot. Polyploid plants have often been used to breed new cultivars because they generally exhibit larger flowers, stronger plants, bigger nutrient storage organs, thicker root systems, richer colors, and stronger disease resistance. So far, many polyploid cultivars have been successfully produced with ornamental plants, such as *Impatiens walleriana* (Hook.). (Ghanbari et al. 2019), *Paphiopedilum villosum* (Huy et al. 2019), *Platycodon grandiflorum* A. DC (Xiang et al. 2019), *Escallonia* spp. (Denaeghel et al. 2018), *Chenopodium album* (Mandak et al. 2018), *Taraxacum kok-saghyz* (Luo et al. 2018), and *Petunia axillaris* (Regalado et al. 2017).

The key to polyploid induction is obtaining plants with stable inheritance. Different approaches have been undertaken to induce autopolyploidization in plants. The effectiveness can be improved by tissue culture; various sources of plant material (e.g. in vitro shoots, axillary buds or other organs, tissues, and cells) for treatment are more suitable for induction techniques than in vivo buds (Predieri 2001). Autopolyploid induction is commonly used in seeds (He et al. 2016; Sadat Noori et al. 2017), stem tips (Vainola 2000; Thao et al. 2003), and pollen (Okazaki and Hane 2005), with lower mutation rates and more chimeras. Chimeras often appear during polyploid induction, which leads to an unstable genetic mechanism and complications associated with measuring multiple chromosomes. By combining chemicals with tissue culture, Wang et al. (2015) successfully induced polyploid plants of *Clivia* using immature embryos and identified them by chromosomal and stomatal observations. Using immature embryos can reduce the number of chimeras, resulting in greater numbers of autopolyploids. However, for some ornamental plants with low seed-setting rates, that mainly rely on asexual reproduction, somatic embryos can replace immature embryos for polyploid induction. Somatic embryos originate from single cells that do not undergo fertilization and produce a large number of plants with high genetic stability; more importantly, embryos are not subject to seasonal restrictions. Recently, polyploids of some plants, including pine hybrid (Sandra et al. 2017), *Ziziphus jujuba* Mill. (Shi et al. 2015), *Pseudostellaria heterophylla* (Ye et al. 2010), and *Vitis vinifera* cv. Mencía (Acanda et al. 2015), have been successfully created using somatic embryos, and these polyploids exhibit improved agronomic traits. Unfortunately, there has been no polyploid induction using somatic embryos in lilies.

Polyploidy has a prominent role in producing premium plants and varieties in the lily industry. The artificial induction of polyploidy was assumed to be of particular importance since polyploid lilies have out-performed their diploid varieties with thicker bulbs, larger flowers, brighter colors and darker leaves. Lily breeders have successfully induced polyploidy in tissue culture, as reported for *Lilium* FO hybrids (Cao et al. 2018), *Lilium*×*formolongi* 'Raizan 3'×*Oriental* hybrid 'Sorbonne' (Zhang et al. 2016), *Lilium lancifolium* (Chung et al. 2015), *Lilium auratum*×*Lilium henryi* hybrids (Chung et al. 2013), and *Lilium* Oriental hybrids (Liu et al. 2011). However, no reports are available on the polyploidization of *L. distichum* and *L. cernuum*.

Therefore, we established a somatic embryogenesis system for *L. distichum* and *L. cernuum* using somatic embryos and scales to induce polyploids by soaking and mixed culture. Combinations of colchicine concentrations and periods of time were compared to select the best treatment combination, and comprehensive morphological observations, stomatal observations and root-tip chromosome counting were used to identify the ploidy of doubling plants. Our results provide a foundation for improving the ornamental value of two wild *Lilium* species, creating new *Lilium* germplasm and improving the reproduction coefficient of these resources.

## Materials and methods

### Plant materials and culture conditions

The *L. distichum* (2n = 2x = 24) and *L. cernuum* (2n = 2x = 24) plantlets used in this study were selected from plants cultured on MS medium every 40 days in a growth chamber (ambient temperature: 25 ± 1 °C) under a 16 h light/8 h dark photoperiod

in our laboratory at the Shenyang Agriculture University. The culture medium was based on MS (Murashige and Skoog 1962) medium with 30 g $L^{-1}$ sucrose, 7 g $L^{-1}$ agar and 4.43 g $L^{-1}$ MS vitamins (M519, Phyto Technology, United States) and was supplemented with plant growth regulators including NAA, picloram, and BA. The pH was adjusted to 5.8 with 1 M NaOH before autoclaving at 121 °C for 15 min. All cultures were grown in a growth chamber under dark conditions in 150 mL glass jars.

## Somatic embryogenesis

Plump and thick scales with strong growth were selected from the tissue cultures of the two lily species. Based on previous research in our lab (Zhang et al. 2016), scales (5 $mm^2$) were cut off the thinner parts of the cultures and incubated in $M_1$ medium (MS medium with 0.41 µmol $L^{-1}$ picloram and 1.07 µmol $L^{-1}$ NAA) for somatic embryo formation. The development of somatic embryos was observed every 5 days, and the somatic embryo induction rate was recorded after 40 days. Well-developed somatic embryos were then inoculated on $M_2$ medium (MS medium with 2.21 µmol $L^{-1}$ BA) to obtain blublets and cultured in the dark to obtain plantlets. After another 40 days, we recorded the bulblet induction rate. Somatic embryo seedling formation was observed every 5 days. Each experiment included 60 explants and was carried out 3×. The somatic embryo induction rate, bulblet induction rate and bulblet multiplication coefficient (BMC) were calculated as follows:

$$\text{Somatic embryo induction rate (\%)} = \frac{\text{No. of somatic embryos}}{\text{No. of scales used}}$$

$$\text{Bulblet induction rate (\%)} = \frac{\text{No. of bulblets}}{\text{No. somatic embryos obtained}}$$

$$\text{BMC} = \frac{(\text{\% bulblets}) \times (\text{Average of somatic embryos})}{100}$$

## Morphological observations of somatic embryogenesis

Starting on the 1st day, samples were taken every 2 days for 80 days from the two lily species (0–40 days in $M_1$ medium, 41–80 days in $M_2$ medium). Ten biological replicates were used for each sample. The samples were fixed in formalin-acetic acid-alcohol (FAA; 90% ethanol, 5% formaldehyde and 5% acetic acid; v/v) for 48 h at 4 °C, dehydrated for 1 h with different grades of ethanol (70%, 85%, 95%, and 100%; v/v) and then transferred to an ethanol-xylene mixture (2:1, 1:1, and 1:2; v/v) for 1 h. The samples were then soaked in a solution of dimethylbenzene for 2 h and embedded in paraffin. The embedded wax blocks were sliced into 8 µm thick sections using a microtome (RM 2245, Leica, Germany). After staining with 0.01% toluidine blue, the wax blocks were observed and photographed under an optical microscope (DM 3000, Leica, Germany).

## Polyploid induction

Somatic embryos and scales were treated with colchicine via two methods. For the soaking method, receptors were immersed in 0% (control), 0.01%, 0.05% or 0.1% colchicine (v/v) for 24, 48 or 72 h at room temperature. Then, the somatic embryos were transferred to $M_2$ medium to obtain plants, and the scales were transferred to $M_1$ medium to obtain somatic embryos. For the mixed culture method, somatic embryos and scales were incubated on $M_1$ and $M_2$ medium, respectively, containing 0% (control), 0.05%, 0.1% or 0.2% colchicine for 10, 20 or 30 days; then, the scales were transferred to $M_1$ medium, and the somatic embryos were transferred to $M_2$ medium. The complete plantlets were transferred to MS medium supplemented with 60 g $L^{-1}$ sucrose ($M_3$ medium) every 40 days. Each experiment included 30 explants and was repeated 3×. The polyploid induction rate was determined as follows:

$$\text{Polyploid induction rate (\%)} = \frac{\text{No. of polyploid plants}}{\text{No. of scales (somatic embryos) used}}$$

## Identification of polyploids

### Chromosome counts

Chromosome numbers were determined from actively growing root meristems. The young root meristems were pretreated at 4 °C for 24 h with a 0.1% colchicine solution, transferred to Carnoy's fixative solution (3:1 ethanol: glacial acetic acid, v/v) for 12 h, dissociated at room temperature for 4 min with 5 mol HCl, and stained with Cabernet dye for 20 min. For each plant, at least ten distinct mitotic phases were observed, and the number of chromosomes was counted.

### Observation of stomata

Fresh leaves of diploid and tetraploid regenerated plants of the two lily species at the same stage were placed on slides to observe their lower epidermal cells. The stomatal density, stomatal length and guard cell length of the diploid and tetraploid leaves were observed and measured by optical microscopy (DM 3000, Leica, Germany). Three replicates were assessed for each form of ploidy.

### Determination of chlorophyll

Chlorophyll was extracted from fresh leaves (0.2 g) from diploid and tetraploid regenerated plants of *L. distichum* and *L. cernuum* with 5 mL of 95% ethanol and 5 mL of 80% acetone under dark conditions for 24 h. The chlorophyll concentration was measured by spectrophotometry (TU-1810, Beijing General Instruments, China). The absorbance was measured at 665 nm and 649 nm with 95% ethanol and 80% acetone as the blank control. Three biological replicates were used for each sample, and the average value was obtained.

### Morphological traits

Twelve-month-old *L. distichum* and *L. cernuum* aseptic seedlings were used for morphological observation in the bulblets, scales, leaves and roots of diploids and tetraploids by vernier caliper. Sterile seedlings with strong growth and well-developed roots were transplanted, and their phenotypes were observed.

### Statistical analysis

The experiments were carried out according to a completely randomized design. The data were expressed as the mean ± standard error and were analyzed by a one-way analysis of variance (ANOVA). Significance was evaluated at the 5% and 1% level using SPSS version 19 (SPSS Inc., Chicago, USA).

## Results

### Somatic embryogenesis

In *L. distichum*, somatic embryogenesis though an indirect pathway resulted in dense yellow embryogenic calli (Fig. 1a). Histological examination showed that the cells initially underwent two or four divisions (Fig. 1f), small globular embryos slowly formed, and the embryos gradually developed into globular embryos with smooth surfaces (Fig. 1g). The globular embryos continued to form torpedo embryos (Fig. 1h). In the central part of the torpedo embryos, well-divided meristematic cells were observed, which constituted the growth center of somatic embryos. At this time, the polarization of embryos was observed. The top ends of the torpedo embryos split rapidly to form a protuberance at both ends and then elongated in the middle and lower regions to form cotyledon embryos (Fig. 1i). Finally, polarization became increasingly evident with the formation of mature somatic embryos (Fig. 1e). The histological examination thus confirmed the occurrence of somatic embryogenesis. The somatic embryogenesis rate of *L. distichum* was 89.6%, and 8 shoots differentiated from each scale. In *L. cernuum*, somatic embryogenesis was similar to that in *L. distichum* (Fig. 1k–t). However, somatic embryos were formed via a direct pathway, i.e. directly from the scales. The somatic embryogenesis rate of *L. cernuum* was 89.6%, and 6 shoots differentiated from each scale.

In the two lily species, two pathways were observed for the origin of somatic embryos, and the main pathway was exogenous origin. The rate of exogenous origin in *L. distichum* was 65%, and that in *L. distichum* was 54%. In *L. distichum*, embryogenic cells first formed on the surface or subsurface of the scales on the 15th day; then, embryogenic calli formed (Fig. 2a–c). The embryogenic cells on the scales formed embryogenic calli on the 21st day (Fig. 2d–f), globular embryos formed on the 40th day, torpedo embryos formed on the 50th day, cotyledon embryos formed on the 55th day, and plantlets formed on the 60th day. In *L. cernuum*, embryogenic cells first transformed into scales directly on the 9th day (Fig. 2g–i), embryogenic cells formed on the surface of scales on the 15th day (Fig. 2j–l), globular embryos formed on the 32nd day, torpedo embryos formed on the 35th day, cotyledon embryos formed on the 40th day, and plantlets formed on the 45th day.

### Chromosome doubling

In *L. distichum* and *L. cernuum*, the somatic embryos and scales soaked in colchicine or inoculated on medium supplemented with colchicine were identified by observing chromosomes in the root tip. By using these methods, we obtained 258 regenerated plants from *L. distichum*, including 198 diploids (2n = 2x = 24) (Fig. 3a), 36 tetraploids (2n = 4x = 48) (Fig. 3b) and 24 aneuploids (chromosome number ranging from 25 to 47). A total of 198 regenerated plants were obtained from *L. distichum*, including 133 diploids (2n = 2x = 24) (Fig. 3c), 28 tetraploids (2n = 4x = 48) (Fig. 3d) and 37 aneuploids (chromosome number ranging from 25 to 47). No chimeric plants were found in either *Lilium* species.

### Effects of different receptors on tetraploid mutagenesis

With the soaking method, the best mutagenesis rate of tetraploids in *L. distichum* and *L. cernuum* (28.57% and 23.08%, respectively) obtained with somatic embryos was significantly higher than that obtained with scales (25% and 20%, respectively) ((Fig. 4, Supplementary Tables S5, S6). On the other hand, in the same treatment, the mortality of

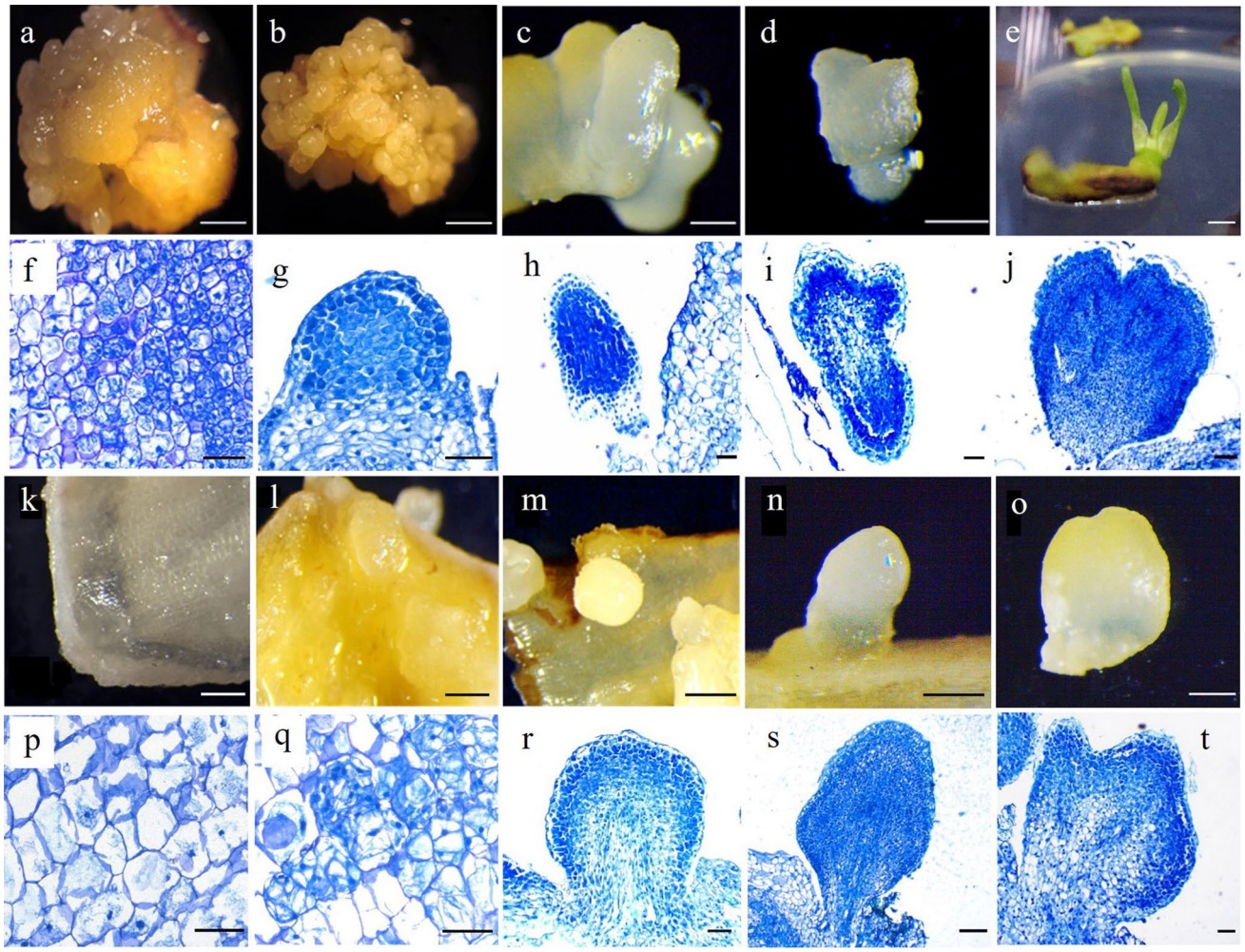


**Fig. 1** Somatic embryogenesis in *L. distichum* and *L. cernuum*. Morphological observations of somatic embryogenesis of the embryogenic callus (**a**), globular embryos (**b**), torpedo embryos (**c**), cotyledon embryos (**d**), and somatic embryo plants (**e**) and histological examination of the embryogenic callus (**f**), globular embryos (**g**), torpedo embryos (**h**), cotyledon embryos (**i**), and somatic embryo plants (**j**) in *L. distichum*; Morphological observations of the somatic embryogenesis of scales (**k**), scales cultured for 2 w that exhibited apophysis (**l**), globular embryos (**m**), torpedo embryos (**n**), and cotyledon embryos (**o**) and histological examination of the embryogenic callus (**p**), globular embryos (**q**), torpedo embryos (**r**), cotyledon embryos (**s**), and somatic embryo plants (**t**) in *L. cernuum*. **a**–**d**, **k**–**o** bar = 500 μm, **f**–**j**, **p**–**s** bar = 50 μm; **e** bar = 50 mm, and **t** bar = 100 μm

scales was higher than that of somatic embryos (Supplementary Tables S1, S2). The results obtained for mixed culture were similar to those obtained with the soaking method. With an increase in colchicine concentration, the toxicity of colchicine to plants increased, the mortality of scales and somatic embryos increased, and the growth status was inhibited to varying degrees. Furthermore, all receptors treated with colchicine germinated later than those in the control group. With the increase in colchicine concentration, the time of receptor germination was delayed, and the lag time increased. The polyploid mutagenesis rate was generally higher when mixed culture was used than when the soaking method was used, but the number of regenerated plants was fewer in mixed culture. The mutagenesis rate was 100% in both *Lilium* species, but only one regenerated plant survived (Supplementary Tables S3, S4).

In summary, polyploid induction using somatic embryos as explants was more effective than was that using scales, either by soaking or mixed culture. Thus, the optimum combinations of treatments were as follows: using somatic embryos as receptors and 0.05% colchicine soaking for 48 h for *L. distichum* (yielding a tetraploid induction rate of 28.57%) and using somatic embryos as receptors and 0.05% colchicine soaking for 24 h for *L. cernuum* (yielding a tetraploid induction rate of 23.08%).

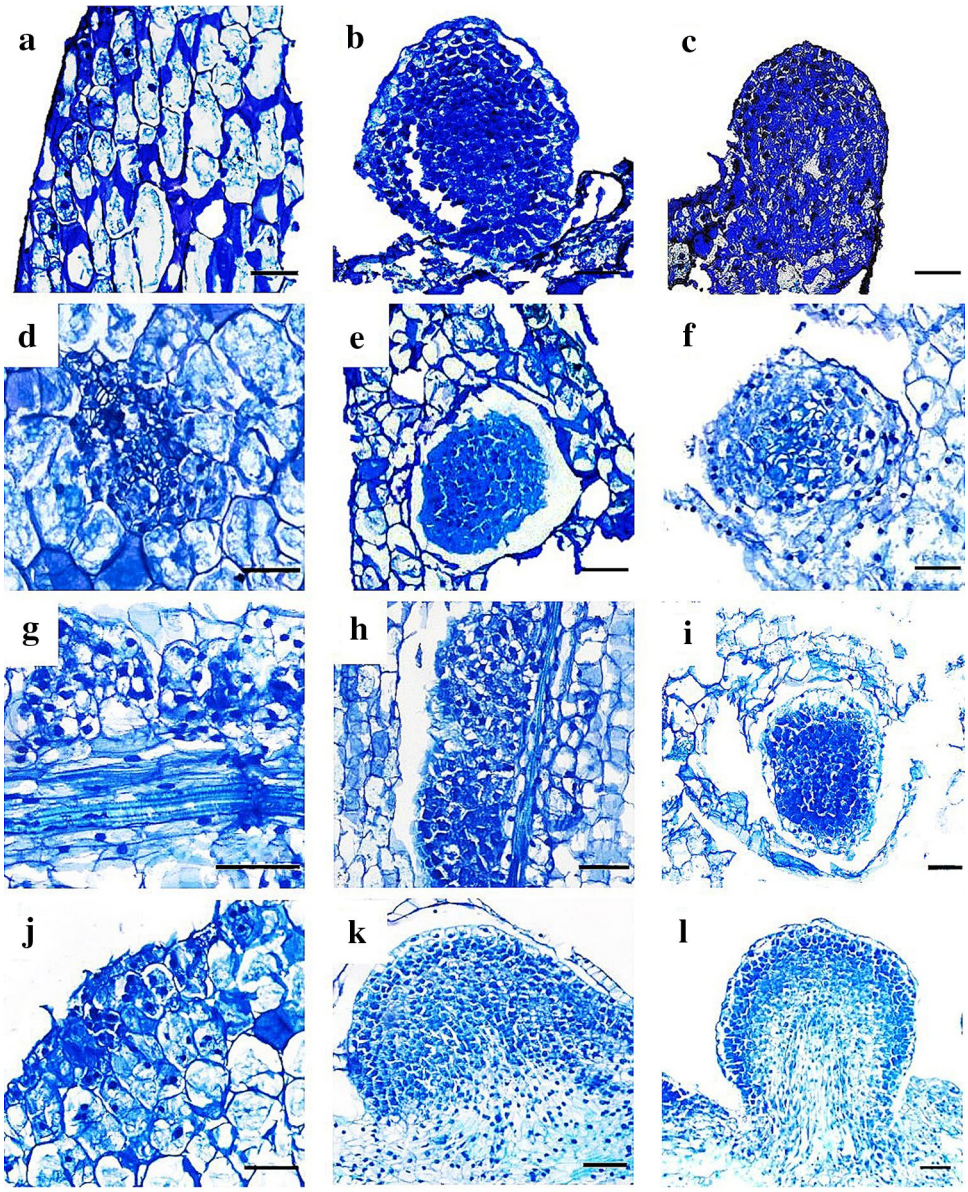


**Fig. 2** Histological examination of the origins of somatic embryos. In *L. distichum*, embryogenic cells first originated from outer cells on the 15th day (**a**), globular embryos formed on the surface (**b**) or subsurface (**c**) of the scales, and then embryogenic cells originated from inner cells on the 21st day (**d**). The entire developmental process from pro-embryo to globular embryo occurred within the embryogenic callus (**e**, **f**). In *L. cernuum*, ducts first formed around the embryogenic cells of scales (**g**), and then embryonic cells originated from inner cells on the 9th day (**h**). Globular embryos were then formed (**i**), and embryogenic cells formed on the surface of scales on the 15th day (**j**–**l**). Bar = 500 μm

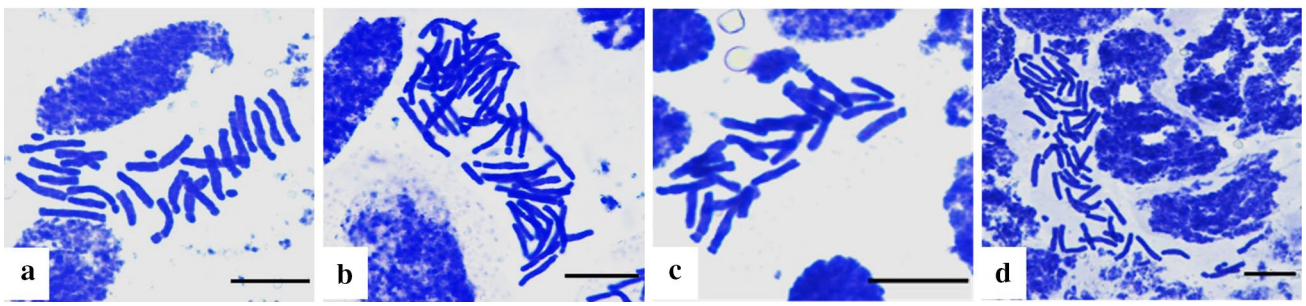


**Fig. 3** Chromosome doubling in *L. distichum* and *L. cernuum*. Diploid plantlets (2n = 2x=24) (**a**) and tetraploid plantlets (2n = 4x=48) (**b**) in *L. distichum*, and diploid plantlets (2n = 2x=24) (**c**) and tetraploid plantlets (2n = 4x=48) (**d**) in *L. cernuum*; bar = 10 μm

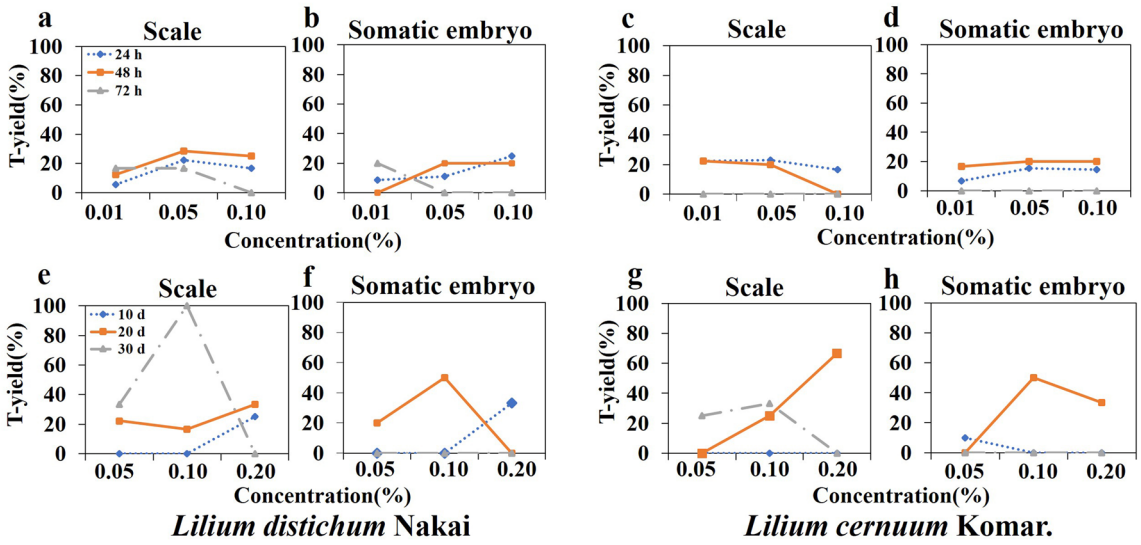


**Fig. 4** Tetraploid yield for both *Lilium* species obtained with the soaking method (**a**–**d**) and mixed culture method (**e**–**h**)

## Morphological characteristics of tetraploid plants

### Leaf characteristics

In *L. distichum*, chromosome doubling resulted in wider leaves, a larger leaf area, and clearer veins, but there was no significant change in the number of leaves (Fig. 6a). In *L. cernuum*, the length and width of leaves changed slightly after chromosome doubling, whereas the number of leaves increased significantly (Fig. 7a–c).

### Stomata and guard cell characteristics

The size of plant organs depends on growth driven by cell division and expansion. Polyploidization enlarges plant cells and organs. In this study, the guard cells of both *Lilium* species were renal guard cells. After polyploidization, as determined from observations of the epidermal cells on the lower surface of the leaves by optical microscopy, the individual stomata became longer, the guard cells increased in size, and the density of guard cells per unit area decreased in tetraploid plants (Fig. 5, Table 1). In addition, the stomatal conductance of tetraploid *L. distichum* increased, whereas that of tetraploid *L. cernuum* decreased.

## Chlorophyll content

In *L. distichum*, significant differences in chlorophyll content were not observed between the diploids and tetraploids, although the chlorophyll content of diploids was slightly higher than that of tetraploids. The leaf color of tetraploids was lighter than that of diploids. In *L. cernuum*, the total chlorophyll content of tetraploid plants and diploid plants was 1.375 and 0.783, respectively (Table 2), and the difference was significant. In addition, the leaf color of tetraploid plants was darker than that of diploid plants.

## Plant morphology

Under the same culture conditions, the tetraploid *Lilium* plants had larger bulbs, thicker scales, wider leaves, and thicker, stronger adventitious roots than the diploid *Lilium* plants. Plants were transplanted into substrate that included turf, vermiculite and river sand (1:1:1, v/v/v). The tetraploids had thicker and wider leaves and significantly more veins than the diploids in *L. distichum*. The leaves were darker and more expanded in the tetraploids of *L. cernuum* than the diploids of this species. Moreover, the tetraploids of the two *Lilium* species were stronger and more adaptable to the external environment than were the corresponding diploids of these species (Figs. 6, 7, Table 3).

# Discussion

## Pathway of somatic embryogenesis

Morphological observation showed that there were two pathways of somatic embryogenesis in two lily species studied. Somatic embryos were formed by the direct pathway in *L. cernuum*. Ducts were formed around the

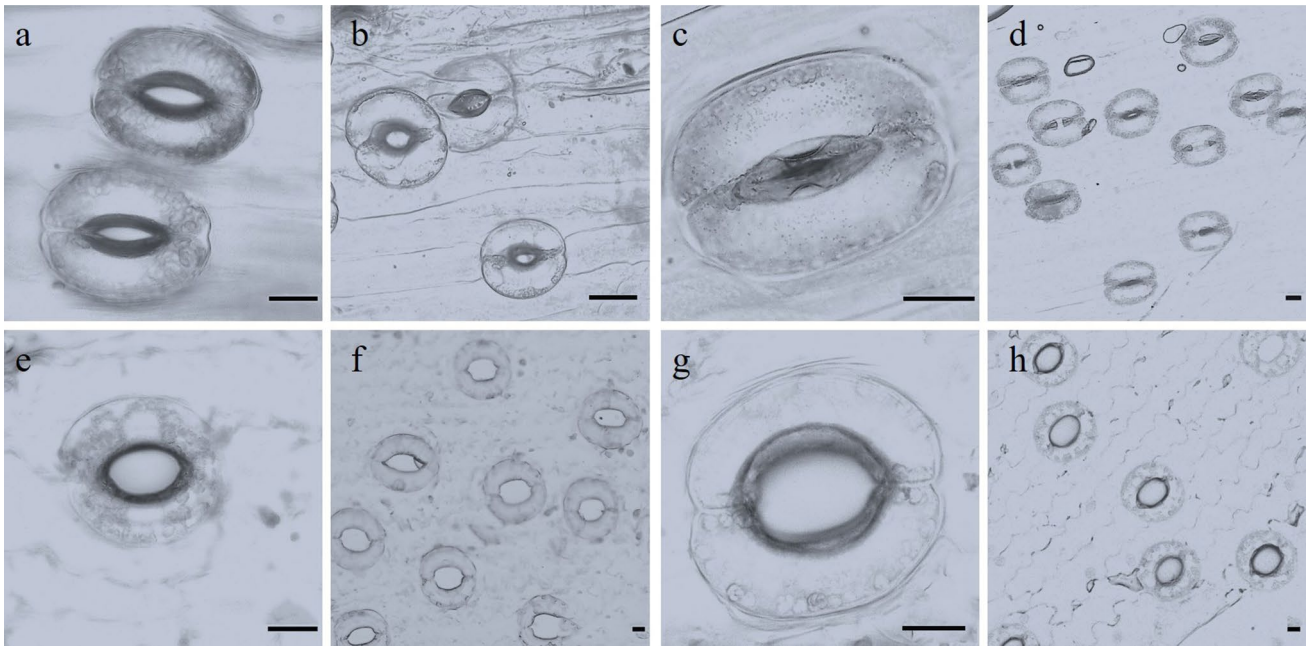


**Fig. 5** Comparison of the stomata and guard cells between diploid and tetraploid plants. Diploid plant (**a**, **b**) and tetraploid plant (**c**, **d**) of *L. cernuum*; diploid plant (**e**, **f**) and tetraploid plant **g**, **h** of *L. distichum*; **b**, **d**, **f**, **h** × 10 magnification; **a**, **c**, **e**, **g** × 40 magnification. Bar = 10 μm

**Table 1** Comparison of stomata and guard cells between diploid plants and tetraploid plants of *L. distichum* and *L. cernuum*

| Sample | Stomatal length (μm) | Guard cell length (μm) | Guard cell width (μm) | Stomatal frequency(number/graph) |
|---|---|---|---|---|
| *L. distichum* | | | | |
| 4x | 89.08 ± 1.85 aA | 139.68 ± 1.39 aA | 50.22 ± 0.64 aA | 11.00 ± 0.50 bB |
| 2x | 54.56 ± 1.56 bB | 76.84 ± 1.15 bB | 27.30 ± 0.38 bB | 25.00 ± 0.88 aA |
| *L. cernuum* | | | | |
| 4x | 90.92 ± 1.30 aA | 105.64 ± 1.32 aA | 40.72 ± 0.88 aA | 7.00 ± 0.58 bB |
| 2x | 45.44 ± 1.05 bB | 56.48 ± 1.54 bB | 22.46 ± 0.45 bB | 16.00 ± 0.65 aA |

Statistically significant events compared with diploids are indicated as normal and capital letters in the table indicate significant differences at 0.05 and 0.01 levels, respectively

**Table 2** Chlorophyll (Chl) contents of plants with different ploidy levels of *L. distichum* and *L. cernuum*

| Sample | Chl a (mg $g^{-1}$) | Chl b (mg $g^{-1}$) | Chl (a + b) (mg $g^{-1}$) |
|---|---|---|---|
| *L. distichum* | | | |
| 4x | 0.61 ± 0.04 aA | 0.27 ± 0.02 aA | 0.88 ± 0.06 aA |
| 2x | 0.63 ± 0.01 aA | 0.28 ± 0.01 aA | 0.91 ± 0.11 aA |
| *L. cernuum* | | | |
| 4x | 1.00 ± 0.05 aA | 0.38 ± 0.01 aA | 1.37 ± 0.08 aA |
| 2x | 0.52 ± 0.02 bB | 0.27 ± 0.01 bB | 0.78 ± 0.02 bB |

Statistically significant events compared with diploids are indicated as normal and capital letters in the table indicate significant differences at 0.05 and 0.01 levels, respectively

embryogenic cells of scales, and a large number of embryogenic cells were formed embryonic cell masses near the ducts; then, somatic embryogenesis was completed. However, this phenomenon was not observed in *L. distichum*, in which somatic embryos were formed by the indirect pathway. We found that somatic embryogenesis was related to the pathway of origin. In the direct pathway, somatic embryos were first formed endogenously. However, somatic embryos were first formed exogenously in the indirect pathway. To our knowledge, this is the first report of the somatic embryogenesis pathway in these two lily species. *Aesculus flava* Sol. (Snezana et al. 2019), *Citrus reticulata* L. (Hussain et al. 2016), and *M. ovalifolia*


**Fig. 6** Morphological observations of diploid and tetraploid *L. cernuum*. **a** Whole plant; **b** bulbs; **c** scales; **e** roots of the diploid; and **f** roots of the tetraploid. **a**, **b** bar = 1 cm; and **c**, **e**, **f** bar = 0.5 cm

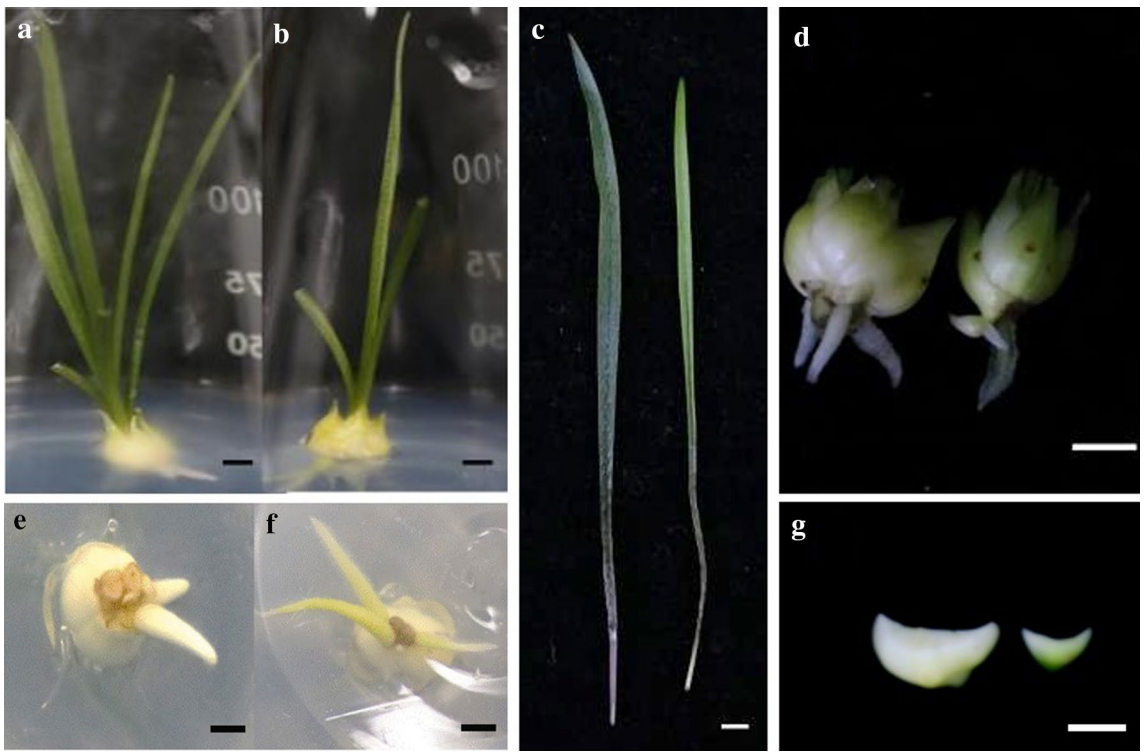


**Fig. 7** Morphological observation of diploid and tetraploid *L. distichum.* **a** Tetraploid plantlet; **b** diploid plantlet; **c** bulbs; **d** leaves; **e** roots of the tetraploid; **f** roots of the diploid; and **g** scales. **a**–**d**: bar = 1 cm; and **e**–**g**: bar = 0.5 cm

(Ouyang et al. 2016) exhibit the same pathway of somatic embryogenesis as that in *L. cernuum.* In *Pistacia vera* L. (Khorzoghi et al. 2019) and *Cucumis melon* L. (Raji et al. 2018), somatic embryos are induced by the same direct pathway as in *L. distichum*. According to the theory of cell totipotency, every cell has the potential to regenerate into a complete plant through somatic embryo. However, not every cell in an embryogenic callus can develop into a somatic embryo (He et al. 2010). Therefore, the different origins of somatic embryogenesis in lily influence the efficiency of somatic embryogenesis.

**Table 3** Morphological characteristics of diploid plantlets and tetraploid plantlets of *L. distichum* and *L. cernuum*

| Sample | Buld circumferential diameter (mm) | Scale thickness (mm) | Leaf wide (mm) | Petiole diameter (mm) | Root diameter (mm) |
|---|---|---|---|---|---|
| *L. distichum* | | | | | |
| 4x | 42.89 ± 1.21 aA | 3.52 ± 0.24 aA | 3.86 ± 0.11 aA | 3.89 ± 0.60 aA | 1.51 ± 0.12 aA |
| 2x | 23.86 ± 0.43 bB | 1.55 ± 0.15 bB | 2.37 ± 0.18 bB | 2.03 ± 0.22 bB | 0.90 ± 0.33 bB |
| *L. cernuum* | | | | | |
| 4x | 23.60 ± 0.36 aA | 2.14 ± 0.05 aA | 3.56 ± 0.17 aA | 3.06 ± 0.16 aA | 2.38 ± 0.60 aA |
| 2x | 12.57 ± 0.37 bB | 0.88 ± 0.14 bB | 1.52 ± 0.12 bB | 1.54 ± 0.10 bB | 1.22 ± 0.27 bB |

Statistically significant events compared with diploids are indicated as normal and capital letters in the table indicate significant differences at 0.05 and 0.01 levels, respectively

## Effects of different receptors on chromosome doubling

Plant receptors play an important role in polyploid induction. Explants are mostly germinated seeds, young root tips, scales of test-tube-grown seedlings, adventitious buds and other tissues with vigorous cell division. In this study, we used both scales and somatic embryos to carry out experiments. The results showed that the somatic embryos of *L. distichum* and *L. cernuum* were the best receptors for inducing polyploids. The highest polyploid induction rates in *L. distichum* and *L. cernuum* were 57.14% and 46.15%, respectively (Supplementary Tables S5, S6), and no chimeras were formed. Therefore, polyploid mutagenesis with the somatic embryogenesis system can reduce or avoid the formation of chimeras, which is a breakthrough in lily polyploid breeding. Denaeghel et al. (2018) obtained tetraploids and chimeras from the buds of three species of *Escalloniales* using oryzalin and trifluralin as mutagens. The frequency of chimeric plants reached 33.3%. Liu and Yang (2015) induced polyploids by adventitious buds in *Lilium koreanum* and achieved a high variation rate of 50%, although the variation rate of chimeras was 38.18%. Tetraploid cells are more unstable than diploid cells, and diploid cells divide more rapidly than tetraploid cells, leading to chimera formation; chimeras seem to have less agricultural value than tetraploids. Somatic embryos originate from single cells without fertilization, and a large number of regenerated plants can be obtained in a short period of time using somatic embryos. Polyploid induction based on the somatic embryogenesis system can not only improve reproductive efficiency but also avoid the production of chimeras. Acanda et al. (2015) induced polyploids of *Vitis vinifera* cv. Menca using somatic embryos as receptors. The highest polyploid induction rate (25%) was obtained at a colchicine concentration of 0.2% and a soaking duration of 24 h, and no chimeras were produced under these conditions.

## Changes in plant morphology after polyploidization

After polyploidization, marked plant morphological changes can be observed. In the two *Lilium* species, tetraploids consistently displayed significantly higher values of bulbs, leaves and the root system than did diploid. Similar trends were commonly observed in tetraploid *Taraxacum kok-saghyz* (Luo et al. 2018), *Brassica campestris* (Baker et al. 2017), and *Echinacea* (Chen et al. 2015). However, increased size in polyploids is apparent not only in their external characteristics but also in their internal stomata, guard cells, chloroplasts, pollen and belowground biomass (Cao et al. 2018; Pollo et al. 2019). Dudits et al. (2016) stated that the size of plant organs depends on the growth driven by cell division and expansion. Polyploidization enlarges plant cells and organs, which is consistent with our results that the stomata became longer, the guard cells became larger, and the stomatal density per unit area was lower in polyploid plants than in diploid plants. In *L. cernuum*, although the number and size of stomata increased, the stomatal conductance decreased. Low stomatal conductance can reduce transpiration and prevent excessive water loss due to transpiration, thus greatly increasing the survival rate of transplants. In our study, we also found that tetraploids were more likely to survive than diploids, which may be closely related to the decrease in stomatal conductance.

## Conclusion

In this study, by morphological observation, we first found the somatic embryo process in *L. distichum* and *L. cernuum*, and confirmed that high numbers of polyploid plants could be obtained by using somatic embryos as receptors. The obtained tetraploid plants exhibited larger bulbs and broader leaves than the diploid and were more adaptable to the external environment. The findings will aid efforts to increase the higher commercial value of *Lilium* and provide better materials for future breeding.

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**Author contributions** HS and LF conceived and designed the experiments. LF, YZ and ML performed the experiments and analyzed the data. CW provided help with the experimental methods and participated in discussions. HS and LF wrote and revised the paper. All authors read and approved the final manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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