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Morphological characteristics of flower organs and *in vitro* micropropagation of male sterile lines of *Lilium* hybrids

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ABSTRACT

Lily pollen causes many problems affecting humans' quality of life and health, such as clothing contamination and skin or nasal allergies. The manual removal of anthers is time-consuming and labour-intensive, and thus, not conducive to commercial production. It is of great importance to produce pollen-free lily varieties with beautiful shapes. In this study, we studied the morphological characteristics of flower organs and polyploids of six pollen-free filial generations of four lily species and their micropropagations. Lily '5-21' is male sterile and produces uncrackable anthers with inactive pollens under natural conditions. Lily '3-12' is unable to produce anthers, because the petals become stamens, thereby leading to infertility. Lilies '3-1', '5-4', '5-35' and '0-0.2' are unable to produce anthers, because they lack floral organs. Polyploids of six hybrid lilies were identified by flow cytometry and chromosome counting, and the results showed that lily '5-4' was triploid and the rest were diploid. Morphological observation indicated that flowering periods were from early July to early August and lasted for about 11 days. Six male sterile lilies had low seed setting rates when they were used as female parents. Most of the seeds were flaky with developed embryos. Therefore, asexual reproduction is optimal for these six male sterile lilies. The male sterility of six lilies was associated with their parents' pollen activity. Scales of '5-4' and '5-35' were used as explants for *in vitro* micropropagation. The findings of the present study lay a foundation for breeding of pollen-free lilies.

Keywords: male sterility, micropropagation, morphological characteristics, pollen-free lily

INTRODUCTION

Lilies (*Lilium* spp.), a group of monocotyledonous ornamental plants, are one of the main bulbous flowers in the flower industry. The lily anther contains a large amount of pollen that will cause clothing contamination and allergy, thereby seriously affecting the quality of life and health of people. Manual removal of the anthers immediately after flowering is time-consuming and labour-intensive, and thus, not conducive to commercial production. Pollen-free flowers do not cause seed formation by self-pollination, saving energy and resources for fruit production and extending the life of the flower. Additionally, the pollen-free characteristic helps eliminate pollen allergens and avoid gene transfer from genetically modified crops into the ecosystem. Thus, breeding of pollen-free lily varieties is an urgent need for promoting cut-flower lily production. In previous studies, new pollen-free varieties were developed mainly through traditional crossbreeding and mutagenesis breeding (Wang et al., 2021). Pollen-free F1 was produced through hybridisation, and a set of pollen-free genetic traits was established. Male sterile mutants were also obtained through radiation breeding (Wang et al., 2019). The bulbs of Asiatic lily were irradiated with ^{60}Co γ-rays, and pollen abortion was found in M1

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plants. In the present study, pollen-less and pollen-free plants were selected by crossing pollen-free hybrids with fertile plants.

It is very important to determine the best period of stigma acceptability for crossbreeding. Stigmas have a sticky surface receptive to pollen and can be divided into two types: dry and wet according to the types of stigmatic exudates (Allum et al., 2007). Stigmatic exudates that are produced by the style are transferred to the stigma surface (Silva et al., 2013) to optimise conditions for retention and germination of pollen (Zheng et al., 2015). The stigmatic exudates from wet stigmas hydrate with pollen to stimulate or inhibit pollen germination, and also function as a nutritive source for pollinating animals (Li et al., 2019). In the fertilisation process of lily, male and female gametes will meet through the style and stigma, which is the site of interaction between pollen and stigma cells (Podwyszyńska et al., 2015; Shao et al., 2015). In the artificial pollination, a reasonable time of higher stigma acceptability is essential for improving the success rate of pollination scientifically and efficiently (Zhou et al., 2014).

Flow cytometry (FCM) and chromosome counting, as well as observation of morphological and physiological characteristics, are widely applied for determining ploidy levels in plants (Ferreira et al., 2021; Wang et al., 2021). Among them, chromosome counting is the most direct and accurate. Most materials for chromosome counting are the tissues at the vigorous cell division stage, including root tips, stem tips and young leaves (Ghotbi Ravandi et al., 2014). The methods for chromosome preparation include conventional tableting pressing, acid lysis and wall removal method, wall removal and low permeability tablet pressing method, and improved wall removal and low permeability method (Fechheimer, 1960). The tablet pressing method was the first common procedure for chromosome counting (Sattler et al., 2016; Javadian et al., 2017), followed by the wall-removing and low-permeability tablet pressing method that has been successfully improved and applied to the identification of chromosome ploidy of many species such as rice, soybean and cotton (Jin et al., 2008). Compared with chromosome counting, FCM has the following advantages for ploidy level estimation: speed, suitability for a large number of samples in a short period of time, materials' limitations being few and requirement for only a small amount of leaf tissue. In contrast to FCM, it is often impossible to tell the ploidy of some species with small chromosomes by chromosome counting. Owing to its advantages, such as high-throughput, accuracy and resolution, negligible destructiveness to plants, and low operating cost per sample, FCM has been extensively used in polyploid and hybrid breeding, seed biology and technology, and *in vitro* cultures to determine the ploidy level (Dolezel et al., 2007).

In the present study, we report four *Lilium* species (three Asian *Lilium* hybrids [AA], namely 'Pollyanna', 'Prato' and 'Pink beauty', and one Oriental *L. henryi* hybrid [OO], namely 'Sorbonne') and six hybrid progenies. We investigated polyploids of six pollen-free filial generations of four lily species using FCM and chromosome counting. Scales of '5-4' and '5-35' were used as explants for *in vitro* micropropagation and expandable propagation. The '5-4' and '5-35' plants were identified as new varieties. The morphological characteristics of flower organs and their propagation *in vitro* were observed and recorded. The findings from exploration of the cytological characteristics of pollen abortion provide a theoretical foundation for the application of male sterility in *Lilium* (Bakhshaie et al., 2016).

MATERIALS AND METHODS

Plant materials

Fourteen lily hybrids belonging to Asiatic hybrids, Oriental hybrids, Longiflorum hybrids and three wild lilies including 'Pollyanna', 'Brunello', 'Prato', 'Cedeazzle', 'Dark Beauty', 'Elite', 'Navona', 'Yelloween', 'Siberia', 'Sorbonne', *L. longiflorum*, *L. lancifolium*, *L. dahurcum* and *L. brouwnii* were used to construct a hybrid offspring population. The pollen-less and pollen-free lily lines were generated by multi-generation screening of the hybrid progenies derived from conventional breeding. 'Pollyanna', 'Prato', 'Pink Beauty' and 'Sorbonne' were identified as parents of pollen-free male sterile lines. Four *Lilium* species (three Asian *Lilium* hybrids [AA], namely 'Pollyanna', 'Prato' and 'Pink beauty', and one Oriental *L. henryi* hybrid [OO], namely 'Sorbonne') and their six hybrids are reported in this study (Supplementary Table 1). Six hybrids were grown at the Northeast Agricultural University's Garden Laboratory, Harbin, China, to produce the pollen-free plants by crossbreeding (Supplementary Table 2). Thirty plants of each lily variety were grown in the Horticultural Experiment Centre of Northeast Agricultural University, Harbin, China.

The scales of hybrid lilies '5-4'and '5-35' were used as explants for propagation *in vitro* on sterile Murashige and Skoog (MS) media supplemented with 8 $g \cdot L^{-1}$ agar (pH 5.8 ± 0.2) under 3,000 lux illumination at 25 °C with a photoperiod of 16 hr light/8 hr darkness. To screen the most effective medium for adventitious bud induction, 20 scales in each group were cut into explants about 0.5 cm^3 in size and then inoculated onto MS media supplemented with different concentrations and combinations of 1-napthaleneacetic acid (NAA) and N6 benzyl adenine (BA) (Supplementary Table 3). All experiments were repeated at least thrice. The induction rates of adventitious buds were recorded at 30 days after culture to determine the most efficient treatment for adventitious bud induction of hybrids '5-4' and '5-35'.

Adventitious buds were cultured on MS basal medium supplemented with different concentrations

and combinations of NAA (0.1 mg · L^{-1}, 0.5 mg · L^{-1} or 1.0 mg · L^{-1}) and BA (0.5 mg · L^{-1}, 1.0 mg · L^{-1} or 1.5 mg · L^{-1}) (Supplementary Table 4) to identify the most effective proliferation medium for adventitious buds of '5-4'and '5-35' seedlings. Three groups were prepared, and each group contained 20 seedlings that were divided into 10 flasks. The proliferation rates of adventitious buds were recorded at 30 days.

Determination of pollen viability

The unopened flower buds of hybrid *Lilium* 'Pollyanna' and '5-21' were bagged and treated with sulphate paper to avoid external contamination of the pollen. The anthers were sampled at the first day of flowering and placed in petri dishes with sulphuric acid paper on the bottom to record natural cracking at indoor temperature. Pollen viability was determined with *in vitro* liquid culture technique. Pollens of 'Pollyanna' and '5-21' were collected at the same time and cultured at 22–24 °C for 12 hr on a liquid solution containing 5% sucrose, 150 mg · L^{-1} boric acid and 20 mg · L^{-1} $CaCl_2$. Pollens were observed under a microscope (Olympus CX31, Japan) at a magnification of 40×. Three fields of view were randomly observed under low magnification to record pollen germination, which was calculated as pollen tube length more than twice of pollen grain length. Differences in pollen germination were identified by analysis of variance (SAS 8.1 software and Microsoft Excel 2007, USA), and mean data were compared using Duncan's multiple-range test ($p = 0.05$).

Stigma receptivity

The stigma receptivity was determined by measuring the peroxidase activity on the nonpollination stigma surface and using the benzidine-hydrogen peroxide method. Thirty plants were randomly selected and labelled for each variety in bud stage. Three flowers were collected for each variety at the same flowering time between 8:00 a.m. and 9:00 a.m. in the morning of the first, second and third days of the opening of the flowers and kept hydrated until the experimental analysis. Then the stigma was cut from the flower with a scalpel and completely immersed in benzidine-hydrogen peroxide solution for 10–15 s to photograph for colouration under the dissecting microscope. The appearance of a blue colour with bubbles on the surface of the stigma indicated stronger stigma receptivity. A deeper blue and more bubbles indicated more stigma acceptability.

Stigma secretion

The number and colouration of the stigma secretion were visually observed during anthesis in 10 varieties.

Observation of morphological characteristics of flower organs

The morphological characteristics of flower organs were continuously observed starting from the beginning of the flowering stage, every day from 7:00 a.m. to 04:00 p.m. at an interval of 4 hr, until the lily flowers withered and fell off. Morphological traits included bud size, petal length, petal width, corolla diameter, specific length of pistil and stamen, flower part colour, morphology, stigma length and style length (Sato and Miyoshi, 2005). The dynamic inflorescence was photographed with a camera. The slices were examined and photographed using a compound optical microscope (Olympus BX51, Japan).

Plant hybridisation

The stigmas of six hybrids ('3-1', '5-4', '5-35', '0-0.2', '5-21' and '3-12') were artificially pollinated with pollens of 'Pollyanna' and shaded with transparent paper bags to prevent cross-pollination. The mature ovaries were harvested to collect seeds. The morphology of seeds from the six pollens was observed. The seeds were cultured on *in vitro* medium.

Observation of chromosomes

The root tips of hybrid lily seedlings at the same growth stage were randomly sampled from 8:00 a.m. to 3:00 p.m. to determine the chromosome ploidy with conventional tableting technology. The young root tips were cut into 1-cm sections and rinsed with distilled water for 5 min. Then tips were placed in the mixture of 8% hydroxyquinoline and colchicine for 24 hr at 4 °C. The materials were rinsed with distilled water and placed in fresh Carnot's fixing solution at a low temperature of 4 °C for 24 hr. The materials were rinsed and placed in a 1 mol · L^{-1} HCl solution that was preheated in water bath at 60°C for dissociation of 5–15 min. Then the materials were hydrolysed and rinsed with water for 3 min. The growing points were picked out with a dissecting needle and placed in the centre of a clean glass slide. After the absorbance of excess water nearby with clean filter paper, acetic acid magenta was added. The tips were stained with the ruby fuchsin staining solution and the modified phenol fuchsin staining solution for about 10 min. Then the tablet was fully pressed to ensure better chromosome dispersion. The state of each chromosome preparation was examined under an optical microscope (Olympus BX-532083). The compression slices with multiple divisions, clear chromosomes, complete morphology and excellent dispersion were used to observe dividing cells with clear division phases and to count the number of chromosomes. More than 10 individuals were observed for each of hybrid lily, and at least 30 complete metaphases were observed during chromosome counting. 'Pollyanna' was used as the control.

Ploidy analysis by FCM

The polyploidy of shoots regenerated from treatment with colchicine was measured as previously described by Galbraith et al. (1983). Briefly, the materials were sampled. Approximately 0.1 g of young healthy leaves at the same growth period was sampled between 8:00 a.m. and 9:00 a.m., and rinsed with distilled water. After

removal of excess water on the surface with filter paper, the leaves were placed in a precooled 5-cm diameter plastic dish, and 1 mL of precooled fixative solution was added to fully immerse the leaves. Then the leaf tissue was quickly chopped with a shaving razor within 1 min and filtered with a 400-mesh cell-sieve nonwoven fabric. The filtered fixing solution was collected with a centrifuge tube and placed in darkness for 30–60 min until green colour appeared in the centrifuge tube. Then 10 µL of 10 mg · mL^{-1} RNAase was added to remove the RNA, and the tube was placed in a 37 °C water bath for 25 min. Then 200 µL of propidum iodide (PI) staining solution was added to the tube and a gentle waving motion was performed to ensure dispersion of the nuclei. The centrifuge tube was placed in darkness at 4 °C for 20 min. Then the filtered isolated nuclei were analysed with a BD FACSAria II (Becton Dickinson Biosciences, San Jose, CA, USA). *L. dazzling* (3×) was used a triploid reference, and *L.distichum* (2×) was used a diploid reference. All measurements were repeated at least thrice.

RESULTS

Pollen characteristics

The pollen characteristics of hybrid *Lilium* '5-21' and diploid *Lilium* 'Pollyanna' were observed under a microscope. The pollen grains of hybrid *Lilium* '5-21' showed a fuller shape, a mostly round or uneven oval shape, an uneven distribution of cytoplasm, and less or no content. Some grains showed partial content overflow, whereas some had no or an unobvious nucleus. Compared with hybrid *Lilium* '5-21', 'Pollyanna' had smaller, full-shaped, and mostly round or oval pollen grains with an obviously visible germination ditch and a hole on the surface as well as substantial contents, thereby showing a higher germination rate (Figure 1). Therefore, we concluded that the hybrid lily '5-21' is male sterile.

As shown in Table 1, the male parents showed pollen germination rates >50%, including as much as 94.87% for 'Prato' and 80.53% for 'Sorbonne'. Therefore, these breeds are suitable for use as the male parents for hybrid combination. It is preliminarily predicted that the segregation of the anther without anther or anther-independent or stamen-lobe is not related to the pollen vitality of the male parents (Table 1).

Stigma receptivity

The stigmas of *Lilium* '3-1' and '5-35' had stronger receptivity in the first 4 days after flowering. Stigma colouration varied greatly. A large number of small bubbles were generated around the stigma first and lasted up to the fifth day after flowering. On the sixth day, the stigma colour became lighter with only a few small bubbles, indicating a gradual decrease of receptive strength. The stigma receptivity completely disappeared by the seventh day (Table 2). Compared with lily '5-4', lily '0-0.2' showed a darker blue stigma with small bubbles in the first 3 days after flowering. During the fourth to sixth days, lily '0-0.2' showed a dark blue stigma with a small number of bubbles, whereas lily '5-4' showed a gradual decrease in the number of small bubbles around the stigma, indicating a sharp decrease in receptivity. On the seventh day of flowering, the receptivity of the stigma had completely disappeared (Table 3).

As shown in Figure 2, the stigmas of lilies '5-21' and '3-12' showed good receptivity with a small number of large bubbles during the first 4 days after flowering.

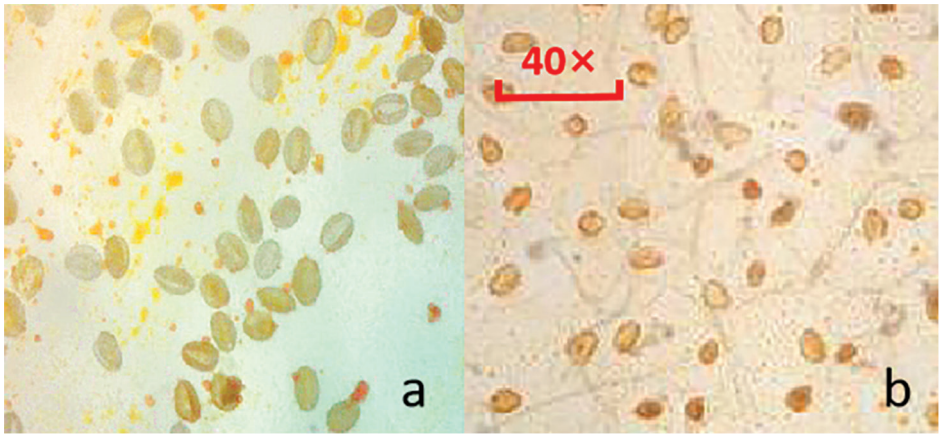


Figure 1. Comparison of pollen germination. (A) Pollen germination of '5-21'. (B) Pollen germination of 'Pollyanna'.

Table 1. The pollen germination rate of the parent lily.

Number	Varieties	Germination rate (%)			Average germination rate (%)
A7	Pollyanna	88.34	86.08	90.22	88.21
A8	Prato	94.87	92.67	93.78	93.77
A9	Sorbonne	80.53	83.72	86.47	83.57

Table 2. Description of stigma receptivity of '3-12' lily.

Genotype	Stigma receptivity at different days after anthesis							
	0	1	2	3	4	5	6	7
'3-1'	–	+	++	+++	++	++	+	–
'5-4'	–	–	–	–	–	–	–	–
'5-35'	–	+	++	++	+	+	+	–
'0-0.2'	–	+	+	++	+	+	+	–
'5-21'	–	+	++	+++	+++	+	+	–
'3-12'	–	+	+++	+++	++	++	+	–

–, no reaction; +, weak positive reaction; ++, strong positive reaction; +++, very strong positive reaction.

Table 3. The relationship between stigma secretion and stigma receptivity.

Genotype	The time at which a secretion began to appear on the stigma (days after anthesis)	The time at which a secretion began to peak (days after anthesis)	The time at which the stigma began to be receptive (days after anthesis)	The time at which stigma receptivity began to peak (days after anthesis)
'3-1'	1	2	2	3
'5-4'	2	4	-	-
'5-35'	1	3	2	4
'0-0.2'	3	4	3	4
'5-21'	2	3	3	5
'3-12'	0	2	3	4

Bubbles were greatly reduced on the fifth day and were almost gone on the sixth day. The stigma receptivity disappeared completely by the seventh day after flowering (Figure 2).

The stigmas of lilies '5-4' and '0-0.2' were dry with no exudation of mucus on the surface and an amount of dry powder. Lilies '3-1' and '5-21' showed higher amounts of mucus oozing from stigmas compared with lilies '5-35' and '3-12'. As shown in Figure 3, their stigmas had receptivity after 2–3 days of flowering, and the wet stigma of the male sterile lily showed more receptivity than the dry stigma.

The horizontal and longitudinal sections of the stigma at the same flowering period showed that stigma heads of lilies '5-21', 'Pollyanna' and 'Prato' were solid with thick and dense surface cells, and they had steeper bulges and longer vascular tissue. In three male sterile lilies '3-1', '5-35' and '0-0.2', the stigma heads were hollow with sparse surface cells. The stigmas were high and visible in the cross-sections of the three sides surrounded by a circle of external tough vascular bundles and epidermal cells. Compared with other species, male sterile lilies '5-4' and '3-12' had a larger stigma volume, relatively dense internal cells, and thick epidermis cells. Obvious fibre bundles were observed on the transection of the stigma. The stigma surface was convex with more exudation cells at the top of the style and a narrower space (Figure 3 and Figure 4).

The horizontal and longitudinal sections of the ovary at the same flowering period showed that lilies '5-4' and '5-35' had a larger ventricular cavity. Each placenta was connected to the centre of the ovary. Lily '5-35' had a

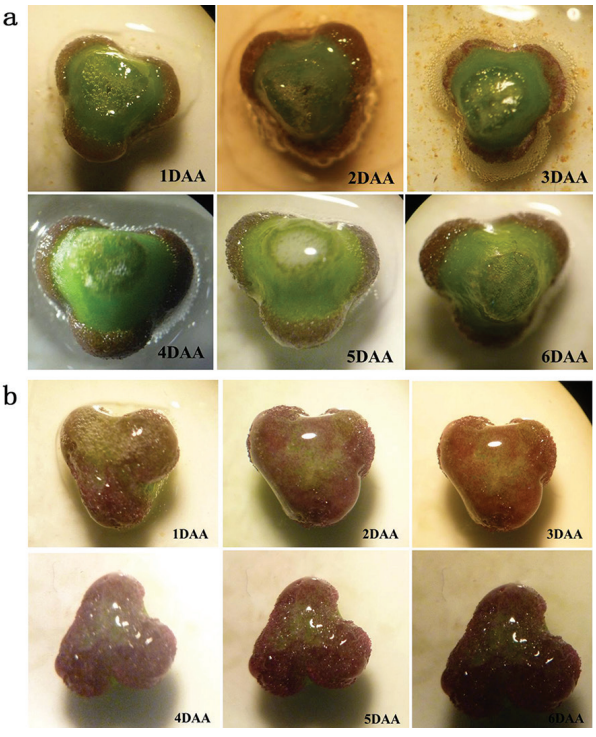


Figure 2. The '5-35' stigmas from 1 to 6 days after anthesis (DAA). (A) Stigmas after treatment with benzidine-H_2O_2 solution. (B) Stigmas showing no reaction but wet secretions..

hollow ovary and three independent placentas. Lilies '0-0.2', 'Pollyanna' and 'Sorbonne' had a fuller ovary with less room for vacancies. The placenta was connected with the centre of the ovary. Lilies '5-4', '5-35',

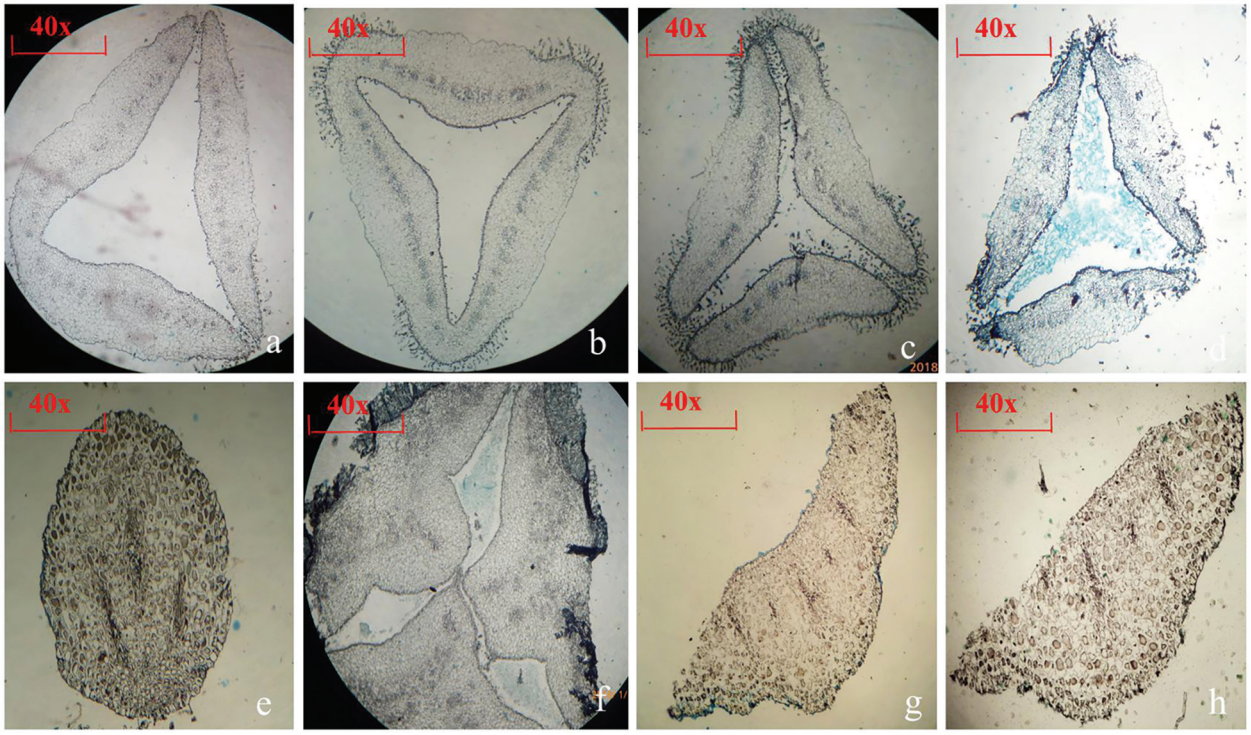


Figure 3. Stigma cross sections. (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12', (G) 'Pollyanna', and (H) 'Sorbonne'.

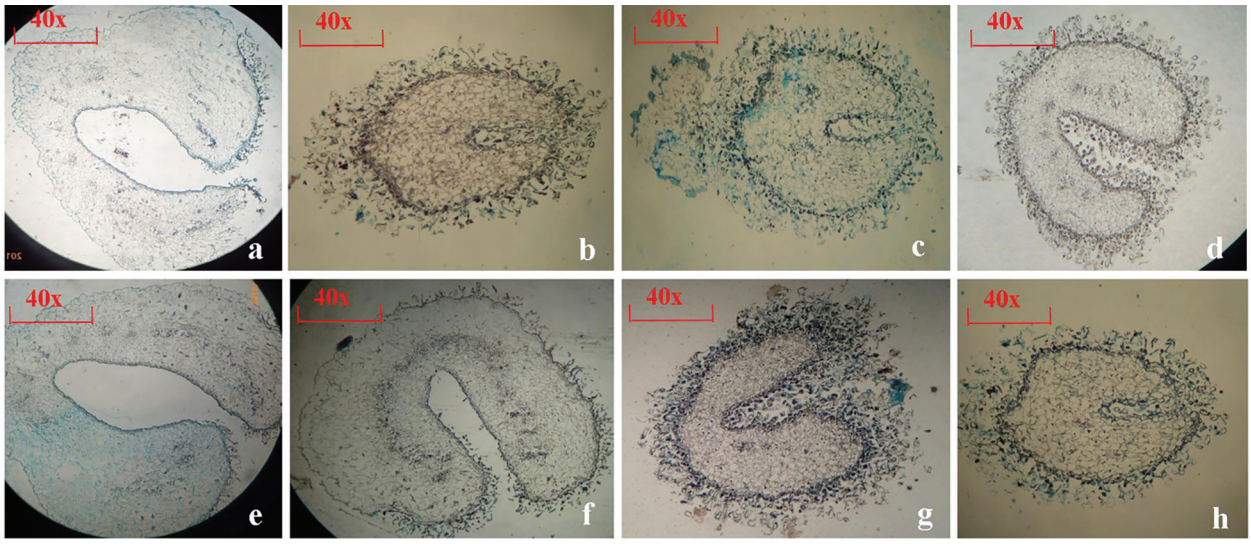


Figure 4. Stigma vertical sections: (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12', (G) 'Pollyanna' and (H) 'Sorbonne'.

'0-0.2', 'Pollyanna' and 'Sorbonne' had obovate ovules. The two ovules of each ovary were bordered in lily '5-21'. Compared with other cultivars, lily '3-12' had smaller ovules. The ovules of two adjacent ovarian cavities were connected by a placenta in a barbed shape. The ovary was hollow with a small cavity, most of which was filled with ovules (Figure 5 and Figure 6).

The longitudinal cross-sections at the same flowering period showed hollow filaments with dense epidermal cells in hybrid lilies. No significant differences were observed among the different varieties. The cavity was biased to one side only in lily '3-12', whereas the filament space was located in the centre of the filament in the other species (Figure 7).

Hybridisation

Six F1s derived from crosses between six male sterile lines and 'Pollyanna' had low seeding rates. Seeds appeared both orange and light yellow with less thickness of flesh (Figure 8, Table 4). The female

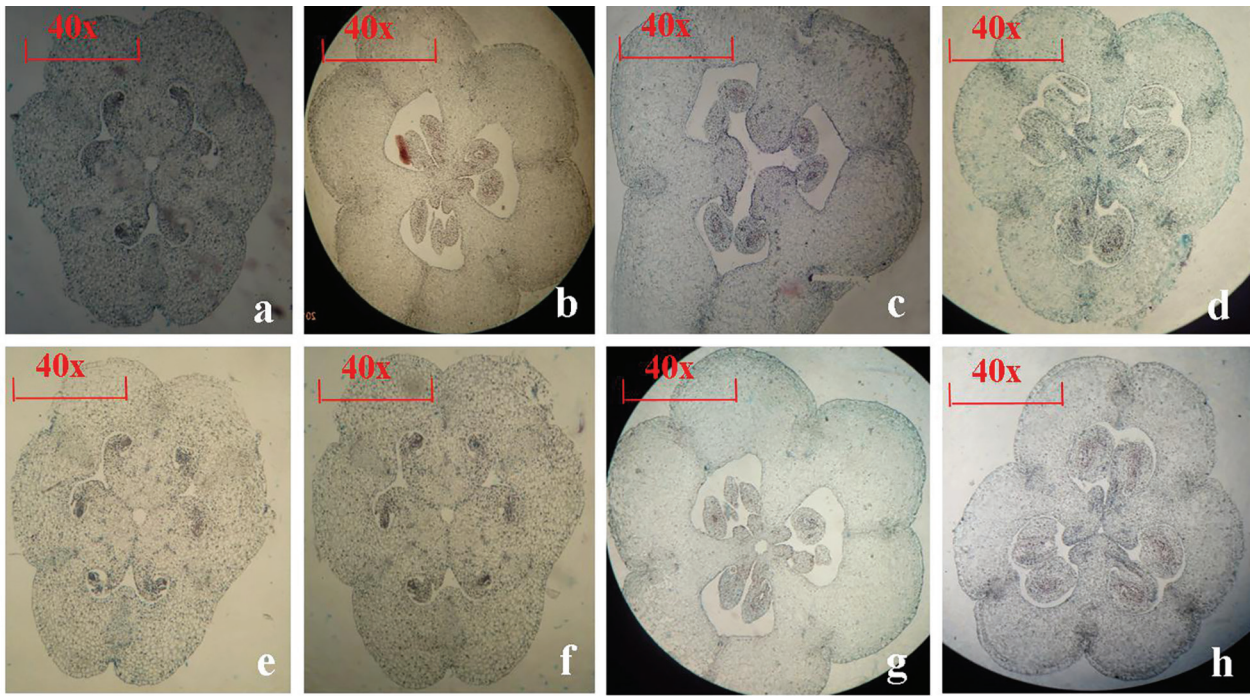


Figure 5. Ovary cross-sections: (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12', (G) 'Pollyanna' and (H) 'Sorbonne'.

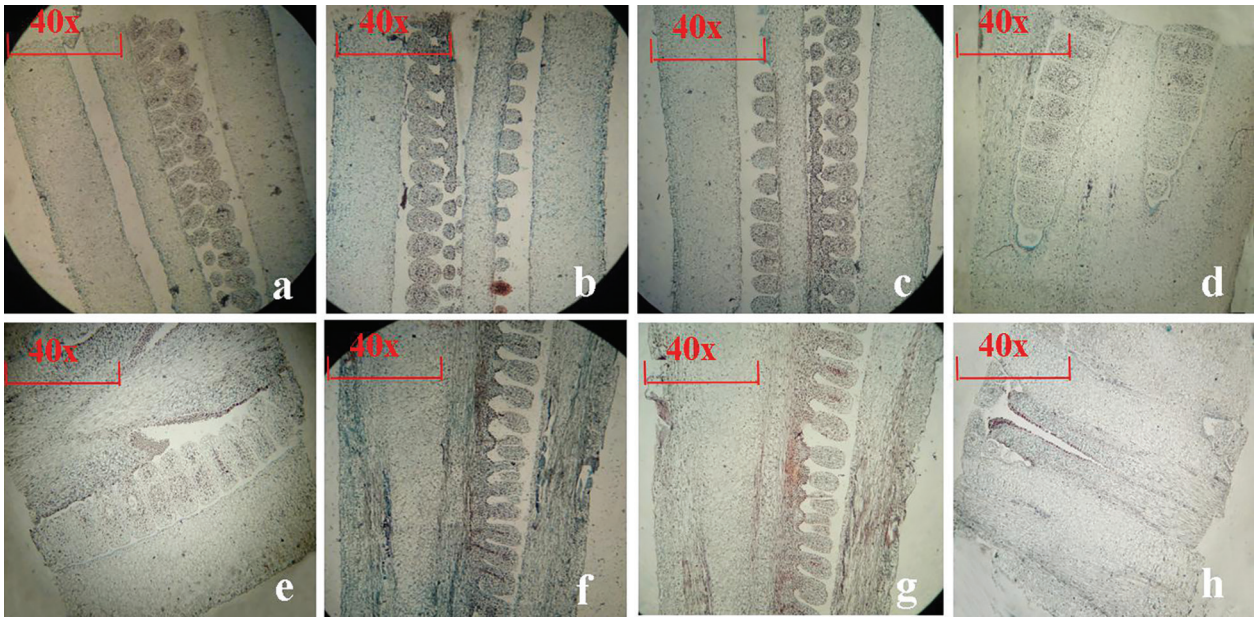


Figure 6. Ovary vertical sections: (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12', (G) 'Pollyanna' and (H) 'Sorbonne'.

parent hybrid lily '0-0.2' produced a small number of flake-shaped seeds, among which only 9% had micro-bulges in the middle, and some were also broken severely. Lilies '3-1' and '5-35' produced 49% and 51% of raised and flaky seeds, respectively, some of which were broken. Lilies '5-21' and '3-12' as female parents produced 61% and 57% raised hybrid seeds, respectively, some of which were broken and raised in the middle. The relationship between receptivity and seed fertility is shown in Table 5. The hybrid '5-4' stigma had no receptivity. Overall, six male sterile lily species had low fertility.

Determination of chromosome ploidy

Chromosome identification methods were compared and analysed, including observation time, dissociation time, and dye and chromosome production technology. The results showed that the best time period for observing

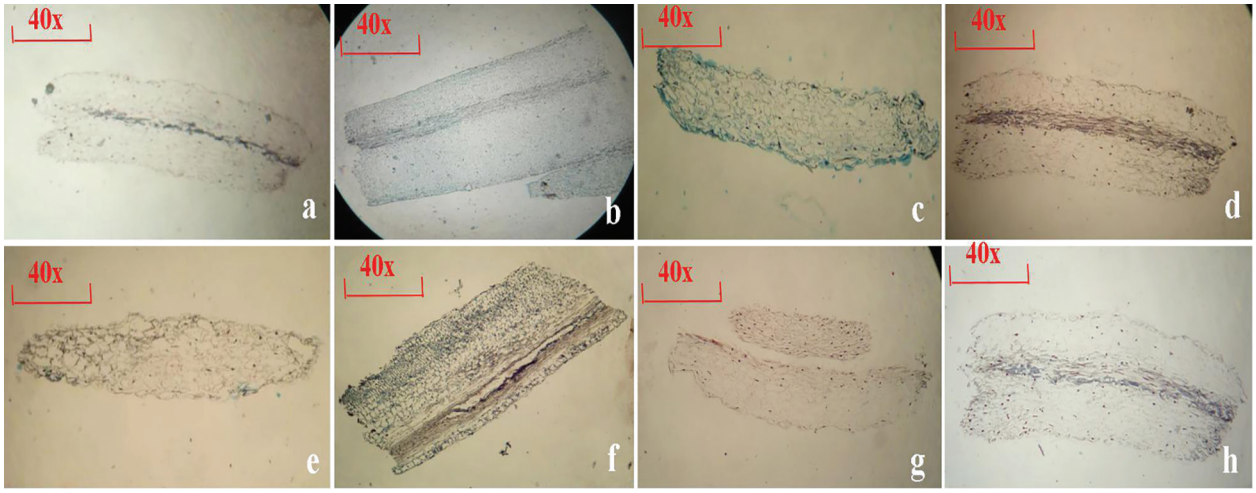


Figure 7. Filament vertical sections: (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12', (G) 'Pollyanna' and (H) 'Sorbonne'.

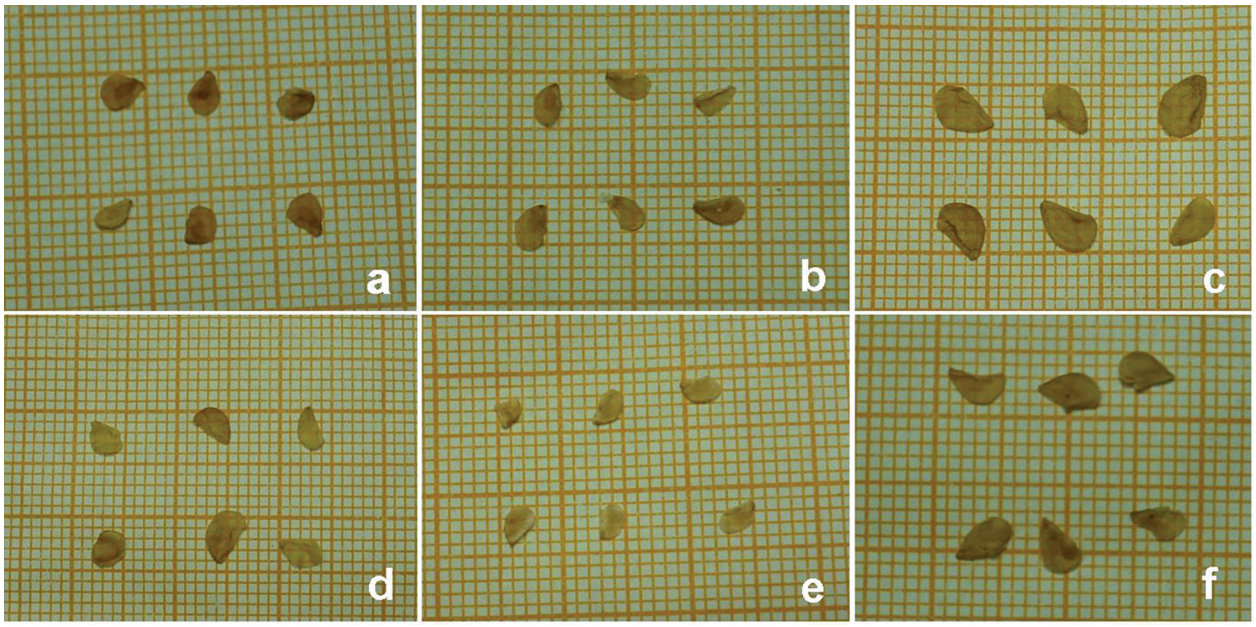
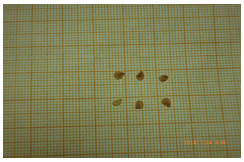
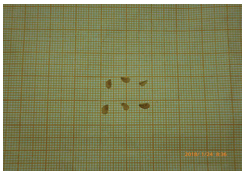
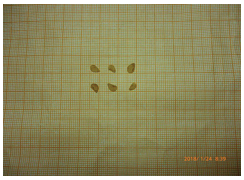
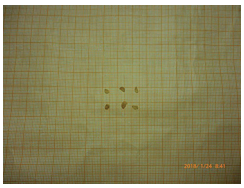
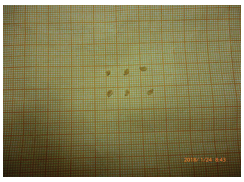


Figure 8. Comparison of seeds of lily: (A) '3-1', (B) '5-35', (C) '0-0.2', (D) '5-21', (E) '3-12' and (F) 'Sorbonne'.

the number of chromosomes in the root tips of hybrid offspring was 8:30–9:00 a.m., when most of the root tip cells were at the peak or middle of division. The best pretreatment was with 0.05% colchicine solution for 24 hr. The optimum apical dissociation time was 7 min for hybrid lilies '3-1', '5-35' and '5-21', 10 min for hybrid lilies '5-4' and '0-0.2', and 5 min for hybrid lilies '3-12' and 'Pollyanna'. Among three dyes, dyeing with Carbo Fuchsin solution (G1165, Solarbio, Beijing) had the best effect and was suitable for image analysis. Staining with a modified phenol solution and acetic acid solution of magenta dye had a poor colouring effect on chromosomes and was not suitable for dyeing hybrid lilies.

The most suitable chromosome preparation protocol for hybrid lilies was as follows: (1) immerse the root tip in distilled water for 5 min; (2) cut about 1 cm from the top of the root tip and immerse it in 0.05% colchicine solution, before pretreatment at 4 °C for 24 hr; (3) rinse with distilled water for 1 min, then treat in Kano's fixed solution for 24 hr, and then dehydrate with 95% ethanol and 85% ethanol for 10 min; (4) store in 70% ethanol for future use; (5) dissociate root tips for 7 min in a water bath at 60 °C during production for hybrid lilies '3-1', '5-35' and '5-21', 10 min for hybrid lilies '5-4' and '0-0.2' and 5 min for hybrid lily '3-12' and 'Pollyanna'; and (6) rinse with distilled water for 1 min before staining with Carbo Fuchsin solution (G1165, Solarbio, Beijing) for 5–8 min and pressing for microscopic examination. Lilies '3-1', '0-0.2', '5-35', '5-21', '3-12' and 'Pollyanna' contained 24(2n) chromosomes, whereas '5-4' had 36 (3n) (Figure 9).

Table 4. The relationship between receptivity and seeding in offspring of plant hybridisation.

Number	Cultivar	Receptivity	Seeding	Picture
1	'3-1'	++	++	
2	'5-4'	–	–	–
3	'5-35'	+	+	
4	'0-0.2'	++	++	
5	'5-21'	+	+	
6	'3-12'	++	++	

–, no reaction or no seeds; +, weak positive reaction or seeds; ++, strong positive reaction or seeds; blank space, no seeds.

Table 5. The seed of filial generation *Lilium*.

Combination	Seeding rate (%)	Seed morphology	1,000 seed weight (g)
A1 × A7	32	Orange, shrivelled	0.017
A2 × A7	36	Yellow, shrivelled	0.021
A3 × A7	26	Yellow, shrivelled	0.018
A4 × A7	39	Bright yellow, shrivelled	0.016
A5 × A7	22	Bright yellow, shrivelled	0.019
A6 × A7	25	Bright yellow, shrivelled	0.017

The ploidy of '5-4' and '5-35' was further confirmed by FCM. *Lilium dazzling* is known to be triploid, whereas *Lilium distichum* is diploid. Triploid showed a single peak on channel 75 (Figure 10A), while diploid appeared on channel 50 (Figure 10B). The results confirmed that '5-4' was triploid (Figure 10C) and '5-35' was diploid (Figure 10D).

Scales of '5-4' and '5-35' were cultured on media supplemented with different concentrations of 6-BA and NAA (Supplementary Table 5) to screen the most convenient medium for clonal propagation. The induction rate (Supplementary Table 5) and growth characteristics (Supplementary Figure 1 and Supplementary Figure 2) were measured after 5 weeks. The results showed that B5 treatment with 1.0 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA was the most effective medium for adventitious bud induction of '5-4' with the highest induction rate of 70.98%. B8 treatment with 1.5 mg · L^{-1} NAA and 0.5 mg ·L^{-1} 6-BA was the most suitable for '5-35' with an induced rate of 72.36%. As shown in Supplementary

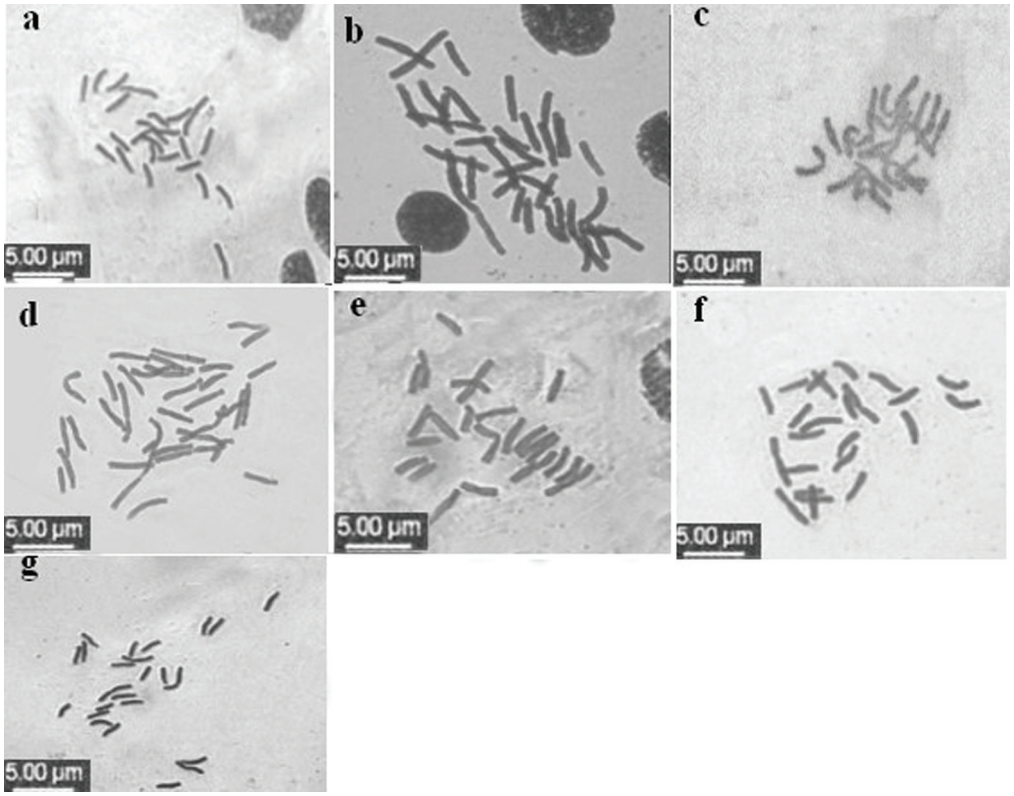


Figure 9. Chromosomal conventional tablets: (A) '3-1', (B) '5-4', (C) '5-35', (D) '0-0.2', (E) '5-21', (F) '3-12' and (G) 'Pollyanna'.

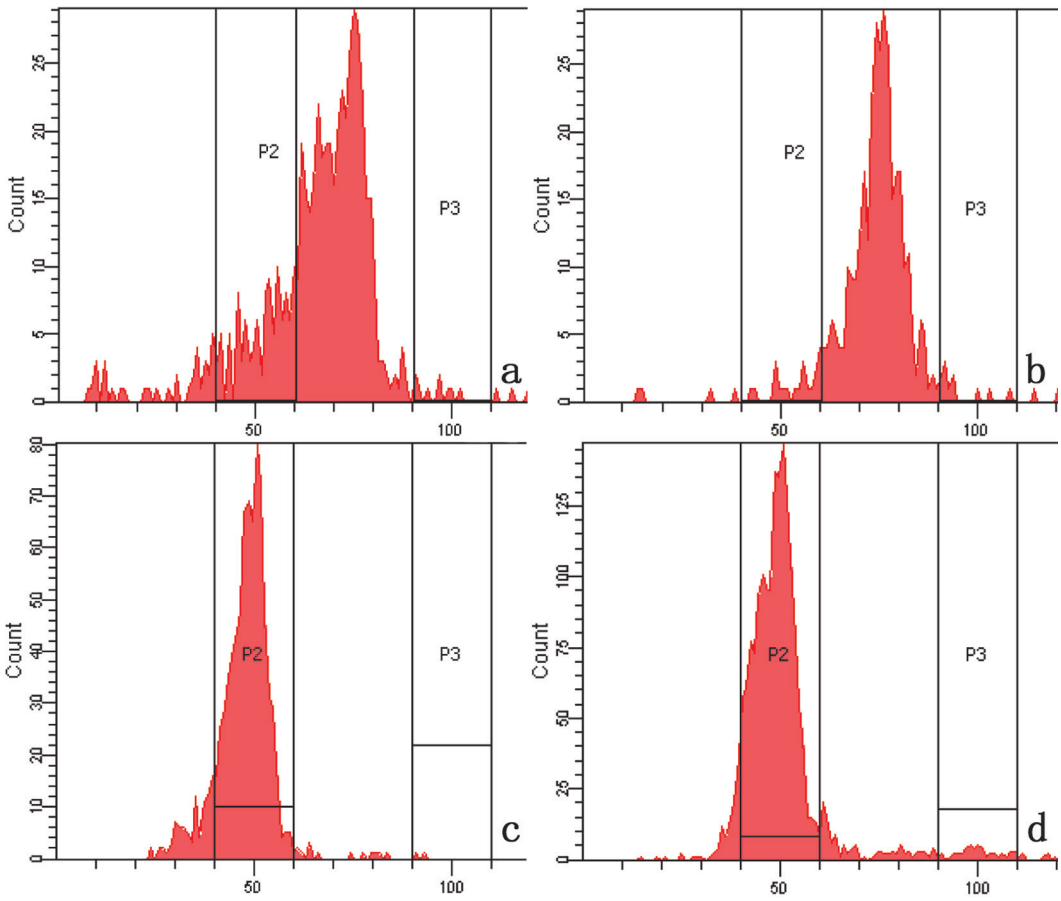


Figure 10. *In vitro* culture initiation and adventitious bud multiplication. (A) Triploid control plants, (B) '5-4', (C) diploid control plants, (D) '5-35'.

Figure 1, compared with control (Supplementary Figure 1A) and all the other groups, '5-4' plants on B5 medium grew vigorously (Supplementary Figure 1F). B8 medium was the best for bud induction of '5-35'. Both '5-4' buds in B5 medium and '5-35' buds in B8 medium showed more bud differentiation points, stronger germs and bigger leaves compared with those grown in other media.

Selection of the best propagation medium

The best propagation medium was screened for '5-4' and '5-35' (Supplementary Table 6 and Supplementary Table 7). Lilies '5-4' and '5-35' showed different growth rhythms. With increasing concentration of 6-BA, the multiplication factor of adventitious buds for '5-4' and '5-35' was raised gradually and reached a maximum with a 6-BA concentration of 1.5 mg · L^{-1}. However, the high NAA concentration had a negative effect on the propagation of both '5-4' and '5-35'. These results suggest that 6-BA was the main factor in the stage of propagation. C8 medium had the highest multiplication factor. The highest multiplication factors were 5.24 for '5-4' and 4.96 for '5-35' on C8 medium. Compared with other media, adventitious buds of 5-4' and '5-35' in C8 medium showed more green leaves, stronger bodies and a higher quantity (Supplementary Figure 3I and Supplementary Figure 4I). Thus, MS medium supplemented with 1.5 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA (C8 treatment) was selected for propagation of both '5-4' and '5-35'. Root induction experiments were performed for '5-4' and '5-35' seedlings (Supplementary Figure 5). Finally, '5-4'and '5-35' were identified as 'Ice Pink Queen' and 'Ice Pure Queen', respectively, by the international cultivar registration authority for the genus *Lilium*. The morphological traits of '5-4' and '5-35' flower are shown in Supplementary Figures 6–13.

DISCUSSION

Study on flower development of male sterile lily

We demonstrated that hybrid lilies '0-0.2', '3-1', '5-35' and '5-4' were male sterile without anthers (Borchert et al., 2009). In the ABC model, stamen petalisation and sterility were caused by the B gene of hybrid lily '3-12', whereas the anthers were unable to be cracked to spread pollen in hybrid lily '5-21' under a natural environment. Pollen is produced internally, but anthers are not vital. This male infertility without cracking ability is one form of functional infertility. Zhou et al. (2014) demonstrated production of aneuploid progenies by crossing male sterile OTO lilies 'Belladonna', 'Candy Club' and 'Travatore' as the maternal parents with two diploid OO cultivars 'Siberia' and 'Sorbonne', and the species *L. regale* Wilson. Different developmental stages of different lily species have different durations. The flowering phenology of plants is affected by weather conditions and nutrition, the latter of which is the main constraint factor. In the present study, the flowering period of male sterile lilies was from early July to mid-August, and flowering lasted about 7 days.

Stigma secretion and stigma receptivity

Stigma receptivity has been studied in only a limited number of ornamental plants (Silva et al., 2013). The morphological changes of the stigma, pollen germination, and staining or enzyme activity detection on the surface have been used to determine stigma receptivity; however, none of these methods can provide accurate estimates (Rejon et al., 2013). Stigma receptivity can be estimated only through controlled pollination and subsequent analysis of effective pollen germination until fruiting. However, this method has not been widely adopted in *Lilium* because of the complex genetic background of different ploidies of modern hybrids and incompatibility of many crosses. In the present study, peroxidase activity of nonpollinated stigma surfaces was examined with a benzidine-H_2O_2 solution, which proved to be a fast, simple and easy method to determine stigma acceptance. Male sterile lily '5-4' had stigma oozing dry powder; however, its stigma had no receptivity in the flowering stage. Therefore, although the exudate was present on the surface of some stigma, it still had no receptivity. Only a firm stigma is receptive to produce firm seeds. However, plants with stigma receptivity may not bear fruit (Jin et al., 2008). In this experiment, the lily '5-4' stigma was receptive but not strong, and the stigmas of the strong plants were all receptive. Pollen viability varies according to plant species, collection time and culture temperature. A coincidence phenomenon of flowering rarely occurs in crossbreeding (Jiang et al., 2013). TTC staining and pollen germination *in vitro* were combined to accurately analyse pollen viability. Male sterile lily '5-21' produced no vital pollen, whereas the other five species had no anther. The stigmas of the six lilies were active from the day before flowering to 3 days after flowering, during which time the relative stigma receptivity was higher and more conducive to pollination. However, successful mating between maternal parents depends on many factors such as the interaction of pollen vigour and style, ovary and the external environment (Lim et al., 2003).

Comparison of identification methods for hybrid ploidy

Different direct and indirect methods have been used to confirm polyploidy, among which indirect methods seem simple but time-consuming. Different morphological and physiological traits, particularly pollen diameter, number of chloroplasts, stomatal size and stomatal density, have been investigated using indirect methods (Moghbel et al., 2015). However, they are unreliable due to environmental effects. Direct methods including chromosome counting have been considered to be effective and reliable but laborious and difficult to operate and observe, especially for plants with high-density cytoplasm composed of large

numbers of chromosomes (Mousavi et al., 2009). They also require highly specific protocols for each species (Allum et al., 2007). Compared with chromosome counting, FCM is more reliable, faster and simpler for analysing large numbers of samples in a very short time (Sattler et al., 2016). FCM is commonly used to quantify DNA in the nucleus and particularly suitable for plants with a small size and ineffective transmission of chromosomes (Xu et al., 2022). Furthermore, FCM can also be used to evaluate large numbers of nuclei (100–10,000 cells · s^{-1}) from different cell layers and types of tissues (Dolezel et al., 2007). Aneuploids have been detected within the progenies of 3x-2x and 3x-4x backcrosses of *Lilium* hybrids (Lim et al., 2003). For some aneuploid plants, FCM has been combined with microscopic chromosome counting to confirm the exact chromosome number (Sun et al., 2004). Somatic aneuploidy also occurred in *Vanilla* hybrids; however, their pollen grains, in contrast to those of *A. officinalis* and *Lilium* hybrids, had an euploid chromosome number (Lepers-Andrzejewski et al., 2011). Therefore, in the present study, we combined chromosome counting with FCM to identify polyploidy. Pan-pan et al. (Javadian et al., 2017) identified ploidy of seedlings with FCM and chromosome counting, and compared the morphological and anatomical characteristics of diploid and tetraploid plants. Javadian et al. (2017) effectively identified polyploids of *Linum album* of the Flax family using chromosome counting coupled with FCM (Javadian et al., 2017). In the present study, we identified that *Lilium* '5-4' was triploid, and *Lilium* '3-1', '3-12', '5-35', '5-21'and '0-0.2'were diploid. Polyploidy *of Lilium* '5-4' and '5-35' was further verified with FCM, confirming that *Lilium* '5-4' was triploid and *Lilium* '5-35' was diploid. Ploidy of '5-4' is different from that of its parent and paternal plants. In addition, cross-breeding studies revealed a hollow upper part of the style that affected the pollen tube extension in male sterile lily '5-4' as a female parent, leading to very low seed setting rate. Most of the embryos were poorly developed, and the seeds were flaky. Further research is needed to investigate the relationship between lily infertility and ploidy.

CONCLUSIONS

In this study, we studied the morphological characteristics of flower organs and polyploids of six pollen-free filial generations of four lily species. Lily '5-21' is male sterile and produces uncrackable anthers with inactive pollens under natural conditions. Lily '3-12' is unable to produce anthers, because the petals become stamens, thereby leading to infertility. Lilies '3-1', '5-4', '5-35' and '0-0.2' are unable to produce anthers, because they lack floral organs. Polyploids of six hybrid lilies were identified, showing that lily '5-4' was triploid and the rest were diploid. Morphological observation indicated that flowering periods were from early July to early August and lasted for about 11 days. Six male sterile lilies had low seed setting rates when they were used as female parents. Most of the seeds were flaky with developed embryos. Therefore, asexual reproduction is optimal for these six male sterile lilies. The male sterility of six lilies was associated with their parents' pollen activity. Scales of '5-4' and '5-35' were used as explants for *in vitro* micropropagation. Finally, the '5-4' and '5-35' plants were generated as new pollen-free male sterile lines. This study demonstrated an entire process for creating new lily varieties.

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AUTHOR CONTRIBUTIONS

J.F. and L.C. designed this project. S.H. and T.Y. carried assays and J.Z. and D.C. performed data analyses. L.C. and S.H. prepared the manuscript. L.C. revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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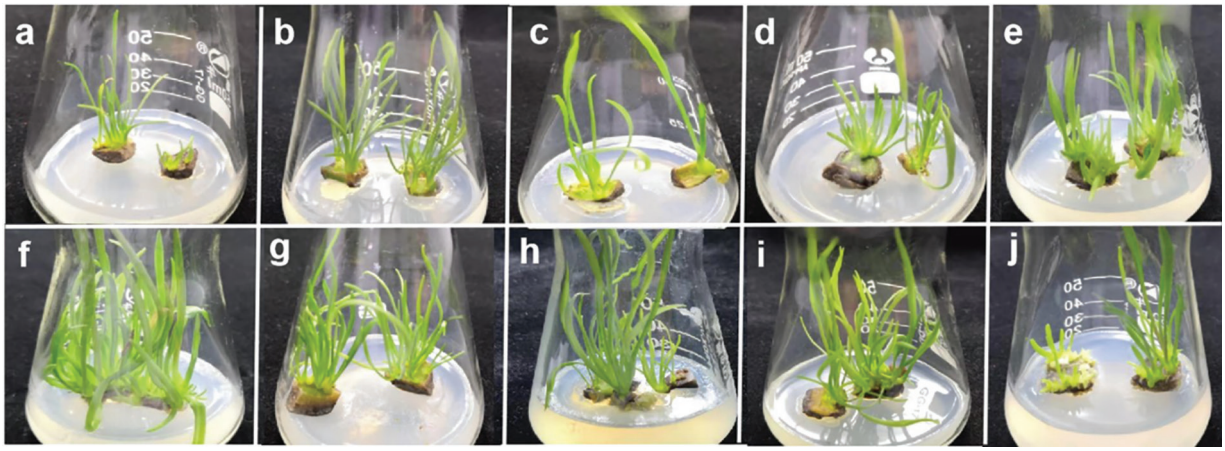
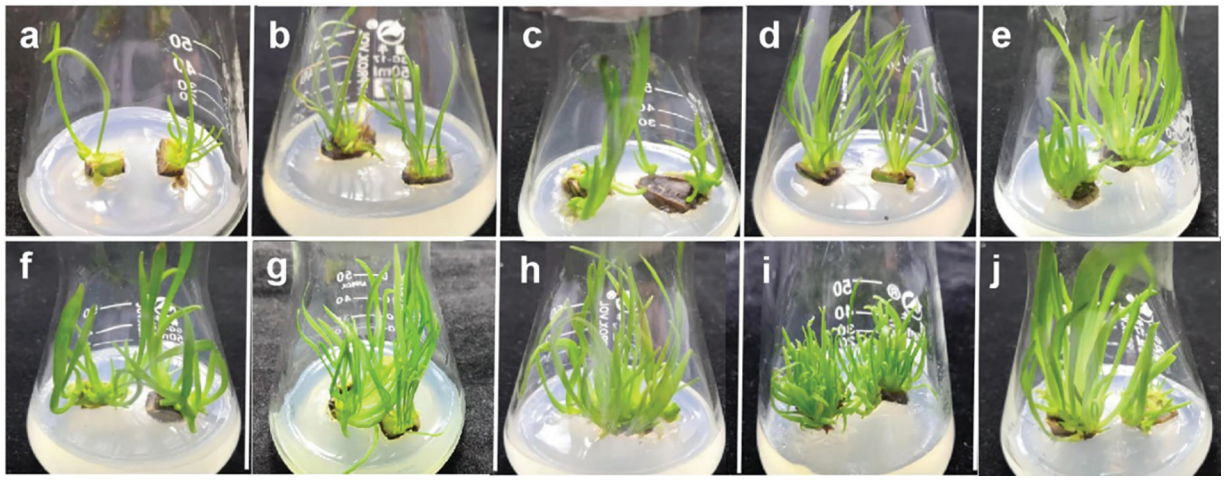
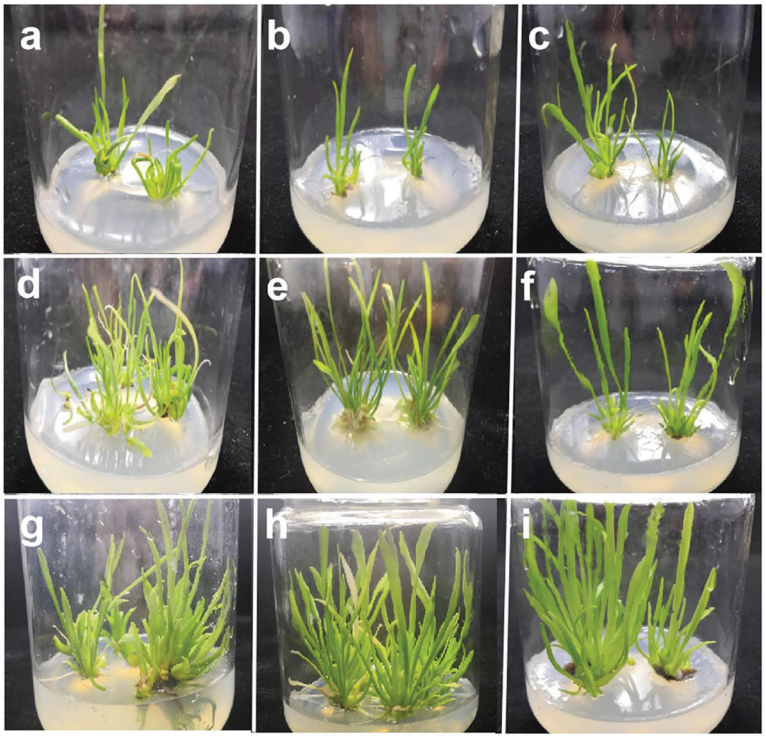
SUPPLEMENTARY MATERIALS



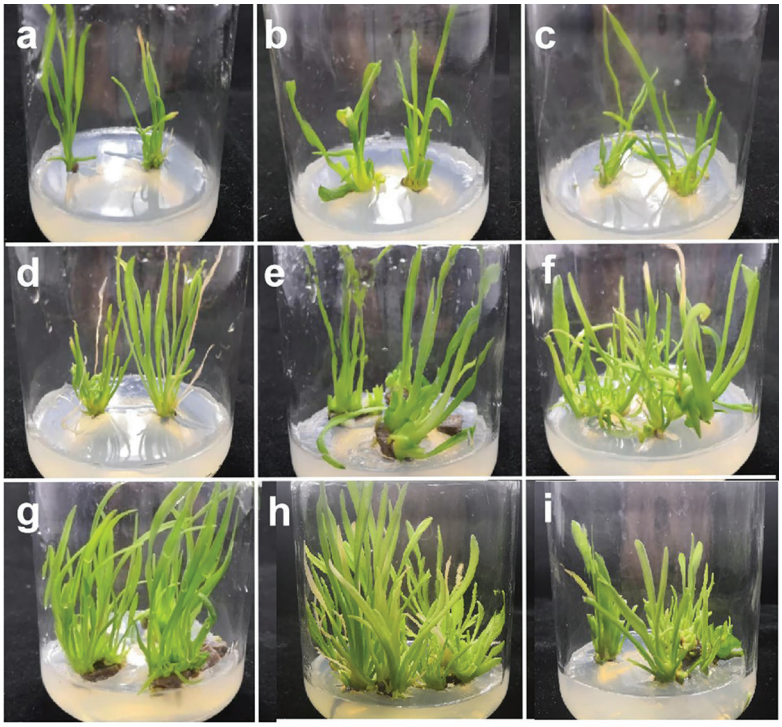
Supplementary Figure 1. Effects of different media on induction of adventitious buds of male sterile lily '5-4': (A) no NAA and no 6-BA; (B) 0.5 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (C) 0.5 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; (D) 0.5 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA; (E) 1.0 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (F) 1.0 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; (G) 1.0 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA; (H) 1.5 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (I) 1.5 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; and (J) 1.5 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA.



Supplementary Figure 2. Effects of different media on adventitious bud induction of male sterile lily '5-35': (A) no NAA and no 6-BA; (B) 0.5 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (C) 0.5 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; (D) 0.5 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA; (E) 1.0 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (F) 1.0 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; (G) 1.0 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA; (H) 1.5 mg · L^{-1} NAA and 0.1 mg · L^{-1} 6-BA; (I) 1.5 mg · L^{-1} NAA and 0.5 mg · L^{-1} 6-BA; and (J) 1.5 mg · L^{-1} NAA and 1.0 mg · L^{-1} 6-BA.



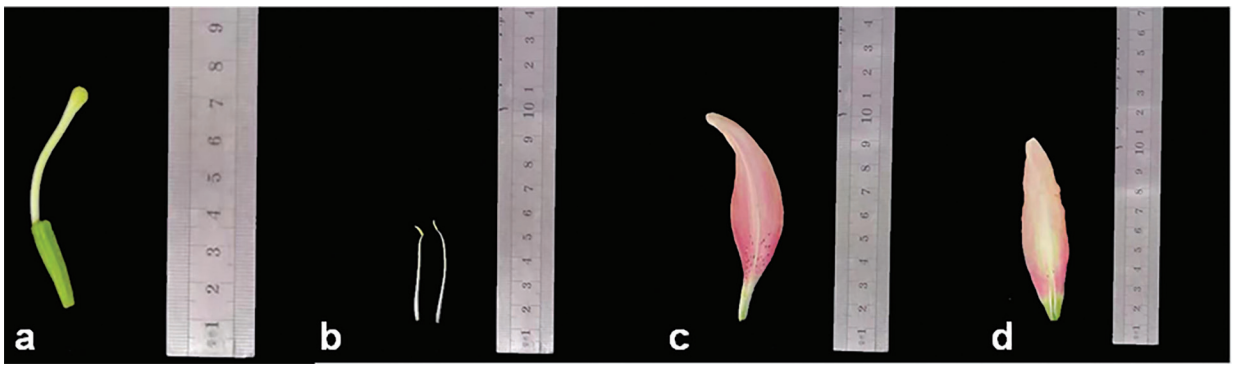
Supplementary Figure 3. Effects of different media on the proliferation of adventitious buds of male sterile lily: (A) 0.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (B) 0.5 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; (C) 0.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (D) 1.0 mg · L^{-1} 6-BA and 0.1 mg · L^{-1} NAA; (E) 1.0 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; (F) 1.0 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (G) 1.5 mg · L^{-1} 6-BA and 0.1 mg · L^{-1} NAA; (H) 1.5 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; and (I) 1.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA.



Supplementary Figure 4. Effects of different media on the proliferation of adventitious buds of male sterile lily '5-35': (A) 0.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (B) 0.5 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; (C) 0.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (D) 1.0 mg · L^{-1} 6-BA and 0.1 mg · L^{-1} NAA; (E) 1.0 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; (F) 1.0 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA; (G) 1.5 mg · L^{-1} 6-BA and 0.1 mg · L^{-1} NAA; (H) 1.5 mg · L^{-1} 6-BA and 0.5 mg · L^{-1} NAA; and (I) 1.5 mg · L^{-1} 6-BA and 1.0 mg · L^{-1} NAA.

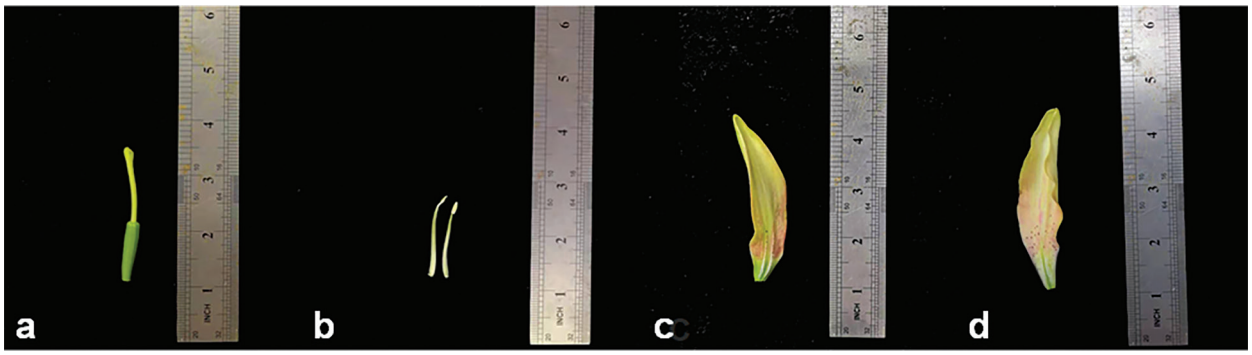
Supplementary Figure 5. Rooting of male sterile lilies: (A) '5-4' seedling and (B) '5-35' seedling.

Supplementary Figure 6. Morphological traits of '5-4' flower.



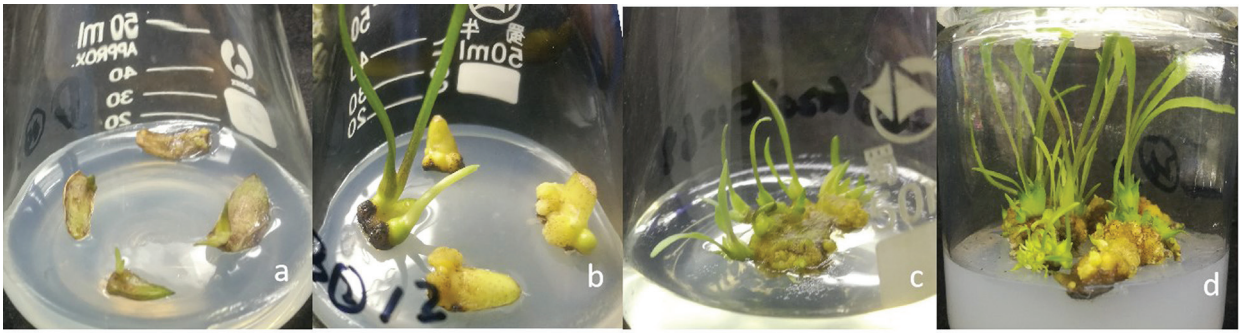
Supplementary Figure 7. The size of petals, filaments, and stigmas of '5-4' flower.

Supplementary Figure 8. Morphological traits of '5-35' flower.

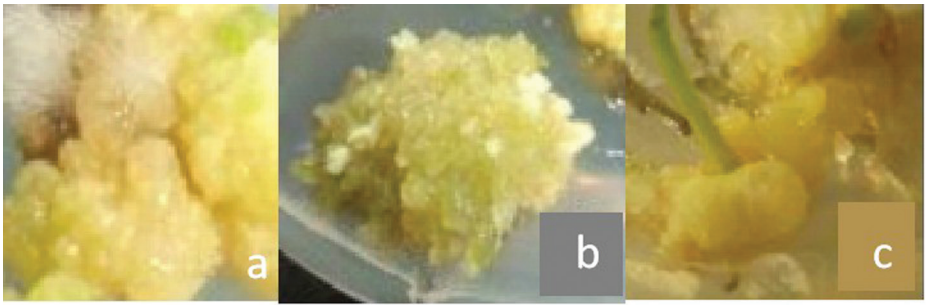


Supplementary Figure 9. Size of petals, filaments, and stigmas of '5-35' flower.

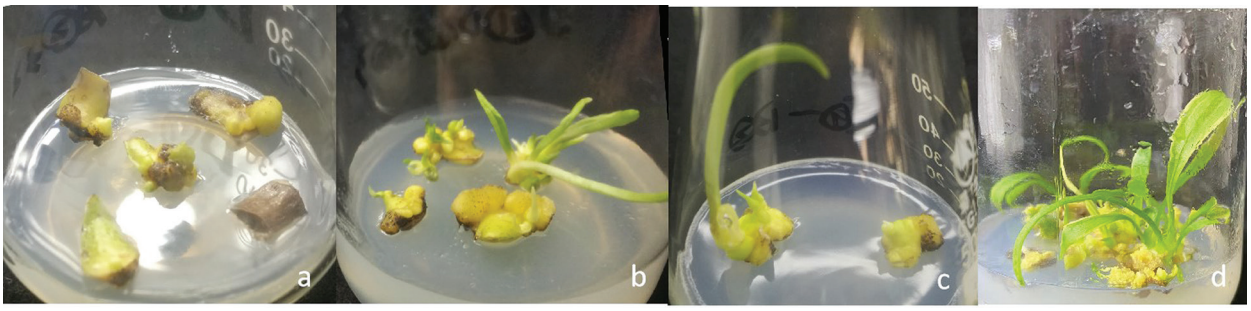
Supplementary Figure 10. Morphological traits of callus of '5-4' *in vitro* cultures.



Supplementary Figure 11. Morphological traits of plantlet of '5-4' *in vitro* cultures.



Supplementary Figure 12. Morphological traits of callus of '5-35' *in vitro* cultures.



Supplementary Figure 13. Morphological traits of plantlet of '5-35' *in vitro* cultures.

Supplementary Table 1. Characteristics and locations of parental cultivars and the sampling times of floral organs.

Cultivar	Series	Ploidy	Plant location	Sampling time	Image
Pollyanna (A7)	AA	Diploid	Harbin	July 2013	
Prato	AA	Diploid	Harbin	July 2013	
Sorbonne	OO	Diploid	Harbin	August 2013	
Pink Beauty	AA	Diploid	Harbin	August 2013	

Supplementary Table 2. Characteristics and locations of hybrid progeny cultivars and the sampling times of floral organs.

Cultivar	Female parent	Male parent	Plant location	Sampling time	Image
'3-1' (A1)	Pink Beauty	Pollyanna	Harbin	July 2017	
'5-4' (A2)	Pink Beauty	Pollyanna	Harbin	August 2017	
'5-35' (A3)	Pink Beauty	Pollyanna	Harbin	August, 2017	
'0-0.2' (A4)	Pink Beauty	Pollyanna	Harbin	September 2017	
'5-21' (A5)	Pollyanna	Prato	Harbin	September 2017	
'3-1' (A1)	Pollyanna	Sorbonne	Harbin	July 2017	

Supplementary Table 3. Effect of different growth regulator combinations on adventitious bud induction.

Treatment group	NAA ($mg \cdot L^{-1}$)	6-BA ($mg \cdot L^{-1}$)
1	0.5	0.1
2	0.5	0.5
3	0.5	1.0
4	1.0	0.1
5	1.0	0.5
6	1.0	1.0
7	1.5	0.1
8	1.5	0.5
9	1.5	1.0

Supplementary Table 4. Effects of different hormone concentration ratios on adventitious bud proliferation.

Treatment group	6-BA ($mg \cdot L^{-1}$)	NAA ($mg \cdot L^{-1}$)
1	0.5	0.1
2	0.5	0.5
3	0.5	1.0
4	1.0	0.1
5	1.0	0.5
6	1.0	1.0
7	1.5	0.1
8	1.5	0.5
9	1.5	1.0

Supplementary Table 5. Effect of different plant hormone concentration ratios on adventitious bud induction of '5-4' and '5-35' scales.

Treatment	Concentration ($mg \cdot L^{-1}$)		No. of explants	Induction rate (%)	
	NAA	6-BA		5-4	5-35
CK	0	0	60	7.33 ± 0.51 h	6.74 ± 0.57 h
B1	0.5	0.1	60	12.69 ± 1.45 g	13.41 ± 1.68 g
B2	0.5	0.5	60	20.67 ± 1.87 f	25.41 ± 1.03 f
B3	0.5	1.0	60	28.49 ± 2.11 e	33.84 ± 1.54 e
B4	1.0	0.1	60	37.94 ± 2.16 d	37.13 ± 2.37 e
B5	1.0	0.5	60	70.98 ± 3.85 a	46.77 ± 2.52 d
B6	1.0	1.0	60	55.25 ± 1.96 c	54.93 ± 2.26 c
B7	1.5	0.1	60	59.66 ± 2.17 b	66.58 ± 2.72 b
B8	1.5	0.5	60	62.41 ± 3.03 b	72.36 ± 3.04 a
B9	1.5	1.0	60	61.56 ± 2.56 b	65.29 ± 2.97 b

Note: Lowercase letters a-h indicate statistical differences at $p < 0.05$ in Student's t-test.

Supplementary Table 6. Effects of different hormone concentration ratios on proliferation of '5-4' adventitious buds.

Treatment	Concentration (mg · L^{-1})		No. of explants	Multiplication factor
	6-BA	NAA		
C1	0.5	0.1	60	1.79 ± 0.15 g
C2	0.5	0.5	60	1.70 ± 0.12 g
C3	0.5	1.0	60	2.28 ± 0.15 f
C4	1.0	0.1	60	3.21 ± 0.28 d
C5	1.0	0.5	60	2.89 ± 0.17 e
C6	1.0	1.0	60	2.36 ± 0.18 f
C7	1.5	0.1	60	4.56 ± 0.25 b
C8	1.5	0.5	60	5.24 ± 0.32 a
C9	1.5	1.0	60	3.77 ± 0.19 c

Note: Lowercase letters a-g indicate statistical differences at $p < 0.05$ in Student's t-test.

Supplementary Table 7. Effects of different hormone concentration ratios on proliferation of '5-35' adventitious buds.

Treatment	Concentration (mg · L^{-1})		No. of explants	Multiplication factor
	6-BA	NAA		
C1	0.5	0.1	60	1.63 ± 0.12 h
C2	0.5	0.5	60	1.69 ± 0.15 h
C3	0.5	1.0	60	2.01 ± 0.09 g
C4	1.0	0.1	60	3.16 ± 0.24 e
C5	1.0	0.5	60	3.48 ± 0.15 d
C6	1.0	1.0	60	2.84 ± 0.17 f
C7	1.5	0.1	60	3.96 ± 0.29 c
C8	1.5	0.5	60	4..96 ± 0.36 a
C9	1.5	1.0	60	4.41 ± 0.33 b

Note: Lowercase letters a-h indicate statistical differences at $p < 0.05$ in Student's t-test.