A Preliminary Study of Apomictic Characteristics of ‘Mianli’ (Pyrus sinkiangensis Yü)

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Abstract. This study investigated the ploidy of ‘Mianli’ with flow cytometry and the traditional chromosome squash technique. Its pollination biology and the occurrence and formation of embryo sacs before and after flowering were observed in paraffin sections to characterize its embryo sacs. The intersimple sequence repeat (ISSR) marker technique was used to test the uniformity of progeny of ‘Mianli’ treatments. The chromosome number of ‘Mianli’ is 2n = 2x = 34. The ploidy results were consistent with those identified by flow cytometry. ‘Mianli’ is male-sterile, and the anatropous ovule has double integuments. ‘Mianli’ can bear fruit normally and produce fertile seeds under the treatments of emasculation with bagging or no emasculation with bagging, but the seed yield is very low and significantly lower than that under artificial pollination or natural pollination. The developmental process of embryo sacs under natural pollination showed that most megasporeocytes develop into mature sexual embryo sacs through meiosis and a few megasporeocytes degenerate. Some sexual embryo sacs continue to develop into embryos after fertilization, and some sexual embryo sacs are aborted. In addition, new aposporous initial cells are generated irregularly at each stage from the emergence of megasporocyte to the end of sexual reproduction or abortion. The observation of the development of embryo sacs under emasculation with bagging showed that after pollination is blocked, mature sexual embryo sacs degenerate, and aposporous mononucleate embryo sacs appear around the degenerated sexual embryo sacs or in the peripheral tissues. Then, the process of proembryonic masses developing into spherical embryo was observed. A genetic uniformity analysis of progeny of ‘Mianli’ using ISSR was performed. The results showed that the progeny population under emasculation with bagging has high consistency at the molecular level, with some plants having full consistency with the female parent’s banding pattern, demonstrating consistency with the maternal genetic characteristics. The progeny under artificial pollination or natural pollination do not have the same banding pattern as the female parent. Because there is no pseudogamy, all of the progeny are true hybrids. In summary, it seems that ‘Mianli’ only has sexual reproduction in the presence of pollen, and only a few ovules are stimulated to undergo apomixis after pollination is blocked.

Apomixis, i.e., asexual reproduction through seeds, is a natural trait of reproduction in plants (Ozias-Akins, 2006). Unlike sexual seed formation, it does not involve the formation of normal female gametes or fertilization; however, it does involve seed formation. Therefore, apomixis enables plants to maintain their genetic characteristics in future generations (Albertini et al., 2010; Koltunow et al., 2013; Pupilli and Barcaccia, 2012). The application of apomixis in hybrid seed production could achieve hybrid genotypic fixation and inexpensive, large-scale hybrid seed multiplication, which has important implications for its use as a tool in plant breeding (Bashaw, 1980). Since Smith first discovered apomixis in Achyranthes bidentata in 1814, the reproductive strategy has been described in ~4000 flowering plant taxa, including representatives of more than 40 families, after evolving multiple times within the flowering plants (Carrman, 1997). Among the monocotyledons and dicotyledons known today, 75% of taxa exhibiting apomixis belong to three families, the Asteraceae, Rosaceae, and Poaceae, which collectively constitute 10% of flowering plants (Bicknell and Koltunow, 2004; Tavera et al., 2015).

Apomixis is an important mode of reproduction and is of great interest because of its potential application in crop improvement (Albertini et al., 2010). The understanding of the bases underpinning apomixis will lead to extending its agricultural applications. In particular, it has been widely studied in fruit crops, such as Garcinia mangostana L. (Ramage et al., 2004), Citrus L. (Wang et al., 2017), Mangifera indica L. (Krishna and Singh, 2007), Juglans regia L. (Cosmulescu et al., 2012), and Malus (Qu et al., 2008). Research of apomictic plants has involved cytobiology, genetics, molecular biology, and applications in breeding and cultivation (Koltunow and Grossniklaus, 2003; Sharbel et al., 2009; Siena et al., 2008; Yang et al., 2017).

‘Mianli’ is a cultivar of the Xinjiang pear (Pyrus sinkiangensis Yü) that belongs to the family Rosaceae (Li and Zhang, 2021). The precise origin of ‘Mianli’ is obscure, but it appears to have been developed in 1987 at the Luntai National Fruit Germplasm Resources Garden of the Xinjiang Academy of Agricultural Sciences. Now, it is mainly distributed in the pear-producing areas of South Xinjiang. In the Korla region, the flowering period and fruit setting of Mianli are usually earlier than that of the main cultivar Korla fragrant pear (Pyrus bretschneideri Rehdi) (Li and Zhang, 2021), which is a typical early maturing cultivar. As an elite germplasm resource for early maturation, ‘Mianli’ has important value in breeding for pear ripening. At present, apomixis has been widely used in Malus rootstock breeding (Sha, 2007); however, there are few reports of apomixis of pear. We have found signs of apomixis in ‘Mianli’ while investigating the biological characteristics of its pollination. To gain a better understanding of its reproductive characteristics, we performed anatomical studies of ‘Mianli’ stamen and pistil development and examined its pollination biology to reveal the types of apomictic embryo sacs and the formation of asexual embryos featured in ‘Mianli’ from a microscopic perspective. Using ISSR molecular marker technology, relevant primers suitable for ‘Mianli’ and its progeny were selected, polymerase chain reaction (PCR) amplification and electrophoresis were performed on the progeny of ‘Mianli’ submitted to different treatments, and the banding patterns of amplified bands were analyzed to detect the genetic uniformity of the apomictic progeny and identify whether the hybrid progeny were true hybrids. A better understanding of the mechanism regulating the reproduction of ‘Mianli’ would provide a reference for the research on pear germplasm resources.

Materials and Methods

Plant materials. The ‘Mianli’ (Pyrus sinkiangensis Yü) material for this experiment was collected from the 20-year-old pear trees preserved in the Luntai National Fruit Germplasm Resources Garden of the Xinjiang Academy of Agricultural Sciences (E84°14’, N41°47’, altitude of 972 m above
Detection of ploidy in ‘Mianli’. Stem tip chromosome preparation was performed from the end of March to mid-April 2020, from 9:00 AM to 11:00 AM. Actively growing stem tips (0.5 cm at the center of stem tip) were cut from ‘Mianli’ and ‘Korla fragrant pear’ and immediately transferred in an aqueous solution of 0.002 mol/L 8-hydroxyquinoline at 4°C for 6 to 8 h and fixed in fixative solution (methanol:glacial acetic acid = 3:1) for 24 h. Stem tips were placed in hypotonic conditions in 0.075 mol/L KCl for 30 min at room temperature (∼24°C); then, enzymology was performed in the mixed solution of 2.5% pectolyase and 2.5% cellulase (adjusted pH, 5–5.5) at 37°C for 3 to 4 h. Next, the samples were lysed in 1 mol/L HCl solution at 60°C for 15 min, and distilled water was added to generate hypotonic conditions for 30 min. Then, the samples were stained with carbol fuchsin for 30 min. Meristematic tissues measuring 0.1 cm was cut, placed on a slide, and stained with toluidine blue. The well-dispersed split phase was selected for observation under the microscope measuring sections with a thickness of 8 to 10 μm, sections were deparaffinized and stained with safranin-fast green, and slides were sealed with neutral gum). Sections were observed under a Nikon Eclipse 80i microscope and imaged using NIS-Elements F 3.0 software.

Flow cytometry was performed using WBP buffer, which is suitable for the separation and extraction of pear nuclei (Loureiro, et al., 2007). The propion iodide staining solution was prepared as described by Tian et al. (2011).

A sample suspension for flow cytometry was prepared using the modified methods of Galbraith et al. (1983), Isuzuaga et al. (2014), and Tian et al. (2011). Fresh young leaves were harvested from each cultivar (≈1 g) during the spring and washed, dried, and placed in a culture dish with prechilled lysis buffer (≈1–2 mL). The leaves were quickly cut into pieces with a sharp blade. The liquid mixture in the culture dish was filtered through a 400-mesh membrane into a control representing normal fertility. From March to April in 2018 and 2019, flower buds were regularly collected from different ‘Mianli’ and ‘Korla fragrant pear’ trees. After the scales were peeled off, they were fixed with a solution containing 30 to 50 ng/μL. The DNA was stored at −20°C. A Tag DNA polymerase, buffer, and dNTPs were purchased from Beijing Golden Biological Technology Co., Ltd. (Beijing, China). Primers were synthesized by Yingjie Ji (Shanghai) Trade Co., Ltd. (Shanghai, China) and based on 100 ISSR primers sequences provided by Columbia University (New York, NY). ISSR amplification with primers in Table 2 was performed for the purified DNA samples according to the following procedure: the PCR reaction system was 25 μL, including 2.5 μL 10 × buffer (MgCl2), 2 μL 2.5 mmol/L dNTPs, 1 μL of each 10 mmol/L primer, 0.5 U of Taq DNA polymerase enzyme, 1 μL template DNA, and 18 μL double-distilled water. The PCR amplification conditions were as follows: 94°C for 5 min (one cycle); 94°C for 1 min; primer annealing (the temperature is shown in Table 2) for 40 s; 72°C for 2 min (35 cycles); and 72°C for 7 min (one cycle). The annealing temperature was usually adjusted according to the Tm of the primer used in the reaction. Amplified products were mixed with bromophenol blue gel-loading dye and analyzed by electrophoresis on a 2% agarose gel using 1× Tris Acetate EDTA buffer pH 8.0 at room temperature. Photographs were taken and clearly interpretable bandings were counted. All patterns generated were repeated at least three times to obtain reproducible data.

Results

Chromosome ploidy of ‘Mianli’. During this experiment, a high-quality 2C peak histogram was obtained by flow cytometry using external standard calibration [coefficient of variation (CV), 3.49–4.57%], and the CV changed within an acceptable specific range (CV <5%). Korla fragrant pear is a known diploid cultivar, and its DNA content histogram is displayed in Fig. 1-B1. ‘Korla fragrant pear’ showed a single peak, and the peak fluorescence intensity was ≈1.0 × 106 (Fig. 1-B1). ‘Mianli’ showed a single peak, and the peak fluorescence intensity was 1.0 × 107 (Fig. 1-A1). These results were consistent with the hypothesis that ‘Mianli’ is a diploid variety. In addition, more than 90% of the chromosomes were well-dispersed and were selected for chromosome counting. The chromosome number of ‘Mianli’ was 2n = 2x = 34 (Fig. 1-A2 and 1-A3), which is the same as that of the control cultivar Korla fragrant pear (Fig. 1-B1 and 1-B2); therefore, ‘Mianli’ male gametes. Sections were observed under a Nikon Eclipse 80i microscope and imaged using NIS-Elements F3.0 software.

ISSR markers. ISSR amplification was performed for 15 progeny after the treatment of emasculation with bagging, for 20 progeny after the treatment of artificial pollination, and for 20 progenies under the treatment of natural pollination.

DNA extraction was performed using a modified CTAB method according to Martínez et al. (2003). Total genomic DNA was extracted from ≈1 g fresh leaf tissue, and extracted DNA was purified with RNase. An ultraviolet spectrophotometer was used to determine the DNA concentration, followed by DNA dilution to a final concentration of 30 to 50 ng/μL. The DNA was stored at −20°C. A Tag DNA polymerase, buffer, and dNTPs were purchased from Beijing Golden Biological Technology Co., Ltd. (Beijing, China). Primers were synthesized by Yingjie Ji (Shanghai) Trade Co., Ltd. (Shanghai, China) and based on 100 ISSR primers sequences provided by Columbia University (New York, NY). ISSR amplification with primers in Table 2 was performed for the purified DNA samples according to the following procedure: the PCR reaction system was 25 μL, including 2.5 μL 10 × buffer (MgCl2), 2 μL 2.5 mmol/L dNTPs, 1 μL of each 10 mmol/L primer, 0.5 U of Taq DNA polymerase enzyme, 1 μL template DNA, and 18 μL double-distilled water. The PCR amplification conditions were as follows: 94°C for 5 min (one cycle); 94°C for 1 min; primer annealing (the temperature is shown in Table 2) for 40 s; 72°C for 2 min (35 cycles); and 72°C for 7 min (one cycle). The annealing temperature was usually adjusted according to the Tm of the primer used in the reaction. Amplified products were mixed with bromophenol blue gel-loading dye and analyzed by electrophoresis on a 2% agarose gel using 1× Tris Acetate EDTA buffer pH 8.0 at room temperature. Photographs were taken and clearly interpretable bandings were counted. All patterns generated were repeated at least three times to obtain reproducible data.
Fig. 1. Chromosome ploidy detection in ‘Mianli’ leaf cells. (A) ‘Mianli’. (B) ‘Korla fragrant pear’.

both are diploid. The results obtained by the two methods were consistent.

**Development of male gametes of ‘Mianli’**

At the beginning of March, in southern Xinjiang, the temperature begins to increase, and the flower buds of ‘Mianli’ begin to sprout. The cross-section of the flower buds shows that the cells in the middle of the anther have differentiated to form a connective composed of vascular bundles and parenchymal cells (Fig. 2-1). Parietal cells begin to divide, from outside to inside, forming the inner wall, middle layer, and tapetum of the connective. As the peripheral cells further differentiate into the pollen sac wall, the sporogenous cells inside the pollen sac undergo division and develop into larger, polygonal pollen mother cells. By this time, the tapetum cells have been formed (Fig. 2-2).

When the pollen mother cells enter prophase I of meiosis, the tapetum cells of ‘Mianli’ show signs of degeneration. Their arrangement becomes loose, their shape becomes irregular, and their nuclei become small until they disappear (Fig. 2-3). The tapetum degenerates and disintegrates in advance, which is the indication for pollen abortion. In contrast, the tapetum cells of ‘Korla fragrant pear’ develop normally and exhibit high metabolic activity, and the tapetum cells do not completely degenerate or disintegrate until the pollen has matured (Fig. 2-4, 2-10, and 2-11). The meiosis of pollen mother cells of ‘Mianli’ and ‘Korla fragrant pear’ is similar before telophase I, and the meiosis of pollen mother cells is normal from prophase I to telophase I (Fig. 2-3, 2-4, 2-5, and 2-9). During mid-March, the meiosis of ‘Korla fragrant pear’ pollen mother cells becomes abnormal after entering the dyad stage. Most of the pollen mother cells appear adhesive and fail to divide during anaphase II of meiosis, and only a few of them enter telophase II (Fig. 2-6). The few pollen mother cells of ‘Mianli’ that enter telophase II display irregular shapes, and these deformed cells rupture (Fig. 2-7) and disintegrate over the course of further development and gradually disappear. At this time, the tapetum cells have completely disintegrated, leaving only the shrunken, hollow anthers with a small amount of residue in the connective (Fig. 2-8); eventually, they are completely aborted. The pollen mother cells of ‘Korla fragrant pear’ undergo normal meiosis, form dyads and tetrads (Fig. 2-10 and 2-11), and, finally, form normal mononuclear microspores (Fig. 2-12).

**Biological observation of pollination of ‘Mianli’ in the field.** Anatomical experiments confirmed that Mianli is a strictly male-sterile cultivar. Therefore, pollen could not be obtained from pistils under no emasculation with bagging treatment; theoretically, the fertile seeds obtained via such treatment should have the same genotype as the female parent and would be the products of apomixis. From 2018 to 2020, the fruit setting rate and the average seed number per fruit were found to be similar between the emasculation with bagging and no emasculation with bagging treatments. Fruit setting rates with these treatments ranged from 11.9% to 25.5%, and the average seed number per fruit was less than 1 (Table 1). The fruit setting rate and average seed number per fruit for these treatments were significantly lower than corresponding values for artificial pollination and natural pollination. The pollen could not be obtained by pistils under either emasculation with bagging or no emasculation with bagging, but some seeds were obtained, and the germination rate of these seeds was 100% (Fig. 3). This indicated that ‘Mianli’ had the ability of apomixis, and its apomixis can occur without fertilization, but at a low incidence. Other experiments are needed to confirm whether pollen grains have a certain stimulating effect on the initiation of division.

The 3-year fruit setting showed that the fruit setting rates and the numbers of seeds per fruit were similar between artificial pollination and natural pollination. Under both pollination treatments, the number of seeds per fruit was between three and six, and the fruit setting rate was between 51.2% and 72.6%, which were significantly higher than those under emasculation with bagging or no emasculation with bagging (Table 1). Pollination significantly increased the fruit setting.
Fig. 3. Seed development of ‘Mianli’. (A) Natural pollination. (B) Artificial pollination. (C) Emasculation with bagging and no emasculation with bagging.

Table 1. Fruit setting characteristics of ‘Mianli’.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fruit setting (%)</th>
<th>Avg seed number per fruit</th>
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<tbody>
<tr>
<td>No emasculation with bagging</td>
<td>14.2</td>
<td>15.3</td>
</tr>
<tr>
<td>Emasculation with bagging</td>
<td>19.6</td>
<td>11.9</td>
</tr>
<tr>
<td>Artificial pollination</td>
<td>64.1</td>
<td>66.5</td>
</tr>
<tr>
<td>Natural pollination</td>
<td>64.1</td>
<td>66.5</td>
</tr>
</tbody>
</table>

rate and the number of seeds per fruit of ‘Mianli’, indicating that pollen has a significant effect on the seed setting rate. Therefore, for the seeds formed by artificial pollination and natural pollination, it can be inferred that either pseudogamous apomixis or hybridization occurred or both apomictic and sexual embryo sacs were formed.

Development of female gametes in ‘Mianli’: Macrosporogenesis and female gametogenesis. The ovule of ‘Mianli’ was the anatropous type with double integument, and the inner integument was composed of three layers of cells; however, the outer integument was composed of multiple layers of cells (Fig. 4-1). Approximately 8 d before the full blooming stage, when the inner integument is elongated and the outer integument primordium is first revealed, an archesporial cell with a large volume, dense cytoplasm, and a large nucleus is revealed, an archesporial cell with a large volume becomes larger, they present vitality and potential, and they are aposporous initial cells (Fig. 4-2, 4-3, 4-4, 4-5, and 4-6). These specialized nucellar cells appear at all stages of sexual reproduction. In some ovules, aposporous initial cells are observed on the day of blooming (Fig. 5-4, 5-5, 5-10, and 5-11) or in the peripheral tissues (Fig. 4-2, 4-3, 4-4, 4-5, 4-6, 4-10, and 4-11) or in the peripheral tissues (Fig. 5-4).

Fruit setting (%) and seed number per fruit.

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<tbody>
<tr>
<td>No emasculation with bagging</td>
<td>14.2</td>
<td>15.3</td>
<td>25.5</td>
<td>0.64</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>Emasculation with bagging</td>
<td>19.6</td>
<td>11.9</td>
<td>0.40</td>
<td>0.40</td>
<td>0.11</td>
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<tr>
<td>Artificial pollination</td>
<td>64.1</td>
<td>66.5</td>
<td>70.8</td>
<td>5.9</td>
<td>5.5</td>
<td>5.87</td>
</tr>
<tr>
<td>Natural pollination</td>
<td>64.1</td>
<td>66.5</td>
<td>70.8</td>
<td>5.9</td>
<td>5.5</td>
<td>5.87</td>
</tr>
</tbody>
</table>

The development of embryo sacs under the treatment of emasculation with bagging. Sexual embryo sacs and surrounding aposporous initial cells are observed on the day of emasculation (1–2 d before flowering). In some ovules, after the degeneration of the megasporocytes, two or three aposporous initial cells in the center of the ovules begin to vacuolize and develop into asexual mononucleate embryo sacs (Fig. 5-1). At ≈2 d after emasculation with bagging, a multinucleate proembryo mass (Fig. 4-14 and 4-15), which then develops into a spherical embryoid body (Fig. 4-16). The development of endosperm begins with the formation of the nucleus of the primary embryo. The primary endosperm nucleus divides rapidly to produce multiple free endosperm nuclei that are scattered in the cytoplasm of the embryo sac, and the nuclei communicate with each other through the protoplasm (Fig. 4-13).

Development of embryo sacs under the treatment of emasculation with bagging. Sexual embryo sacs and surrounding aposporous initial cells are observed on the day of emasculation (1–2 d before flowering). In some ovules, after the degeneration of the megasporocyte, two or three aposporous initial cells in the center of the ovules begin to vacuolize and develop into asexual mononucleate embryo sacs (Fig. 5-1). At ≈2 d after emasculation with bagging, multinucleate asexual embryo sacs beside the mature sexual embryo sacs are observed in some ovules (Fig. 5-2). At ≈4 d after emasculation with bagging, the embryos and endosperms of the sexual embryo sacs do not initiate division; therefore, they are aborted, which means the end of sexual reproduction (Fig. 5-3). After the end of sexual reproduction, asexual embryo sacs develop, forming asexual mononucleate embryo sacs (Fig. 5-4). At ≈10 d after blooming, the polar nuclei of most ovules do not initiate division, whereas the egg cell at the micropylar end forms a proembryo, and there is a large and obvious polar nucleus near the proembryo (Fig. 5-5). As a result, only proembryos and...
bryo development (Fig. 5-7). Only ovules have free endosperm nuclei, but no em-
ferentiation of proembryos often stop. Some to provide nutrition, the development and dif-
ervation of embryos and endosperms (Fig. 5-8). Therefore, under the treatment of emasculation and the development of endosperms, new aposporous initial cells (ai) are differentiated close to the mono-
nuclear embryo sac (es) (7 d BFB). (5) Binuclear embryo sac (es) containing two aposporous initial cells (ai) (5 d BFB). (6) Normally, there are one to three aposporous initials cell (ai) are differentiated close to the selected spore (6 d BFB). (4) An aposporous initial cell (ai) is differentiated close to the mono-
nuclear embryo sac (es) (7 d BFB). (5) Binuclear embryo sac (es) containing two aposporous initial cells (ai) (5 d BFB). (6) Greatly extended tetranuclear embryo sac (es) containing two aposporous initial cells (ai) (4 d BFB). (7) Octonuclear embryo sac (2 d BFB). (8) Maturing embryo sac containing three antipodal cells (ant), two central cells (cc), and egg apparatus (ea) (1 d BFB). (9) Maturing embryo sac containing antipodal cells (ant), polar nuclei (pn), and an elongated egg cell (ec) (2 d BFB). (10) Macrosporocyte disintegration and multiple active nucellar cells (6 d BFB). (11) Multi-embryo sac develop-
ment (6 d BFB). (12) Pollen tube (pt). (13) There are only primary endosperm nuclei (arrow) and no proembryo in the embryo sac. (14) The egg nucleus divides to form a multinucleated proembryo mass (5 d AFB). (15) The embryo sac has only a proembryo. There is no endosperm development. (16)

The egg nucleus in the embryo sac gradually developed into a spherical embryo and free-nuclear endosperm are present (arrow) (6 d AFB). A = sporogo-
nium; it = integument; ai = aposporous initial cell; mmc = macrosporocyte; fm = functional megaspore; dm = degenerated megaspores; es = embryo sac; ant = antipodal cell; cc = central cell; ec = egg cell; sy = synergid; ea = egg apparatus; pn = polar nuclei; aes = aposporous embryo sac; des = degenerated embryo sac; em = embryo; pt = pollen tube; d BFB = days before full bloom; d AFB = days after full bloom. Modified from Drews and Koltunow (2011). All figures were arranged with the micropylar end facing down.

The observation of ‘Mianli’ embryo sac sections showed that during the process of ovule development, new aposporous initial cells irregularly emerge at each stage from the emergence of the megasporocyte until the end of sexual reproduction or abortion. Generally, there are one to three aposporous initial cells. Therefore, two to three vacuolated aposporous initial cells can be observed in one ovule (Figs. 4-10, 5-1, 5-2, and 5-4). In the samples at different periods after emasculation, the existence and development of mul-
tiple aposporous initial cells are observed and, in some ovules, two proembryos that have spontaneously divided from egg cells are found close to each other at the micropylar end (Fig. 5-5). However, the seeds with a twin embryo are not found in the seeds obtained under emasculation with bagging. We speculated that when multiple aposporous initial cells occur and develop at the same time, the lack of space and nutrition will lead to the abortion of all seeds in the ovule because they have the same viability.

**ISSR assessment and analysis.** ISSR PCR amplification was performed for 57 samples of ‘Korla fragrant pear’ and ‘Mianli’ and their progeny (Table 2). A total of 31 loci were generated after amplification, and the number of DNA bands amplified by each specific primer was be-
 tween 4 and 10, with an average of 7.75 and a length of 250 to 5000 bp. There were 28 poly-
morphic loci, accounting for 91.25% of the loci. Among them, the primers with the fewest poly-
morphic bands after amplification were UBC 836
 and UBC 845, and the number of polymorphic bands after amplification of two other primers is the same at nine. The bands could be divided into three types: common bands of both parents; bands of one parent; and specific bands.

no endosperm form in this type of embryo sac (Fig. 5-6). Because there is no endosperm to provide nutrition, the development and dif-
ferentiation of proembryos often stop. Some ovules have free endosperm nuclei, but no em-
bro development (Fig. 5-7). Only \( \approx 4\% \) of the ovaries exhibit the formation of embryos and the development of endosperms (Fig. 5-8).

Therefore, under the treatment of emasculation with bagging, only a small proportion of the embryos and endosperms of the ovary can spontaneously divide and eventually form normal seeds; this proportion is broadly consistent with that of the field tests. Of the embryo sacs of ‘Mianli’ without pollination and fertilization, a certain proportion go through the normal de-
velopment of embryos and endosperms, indicating that apomictic embryos and endosperms can be formed without fertilization; however, this ratio is low (<5%).
Table 2. ISSR primer amplification results.

<table>
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<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Optimum annealing temp (°C)</th>
<th>Amplified total bands</th>
<th>Polymorphic bands</th>
<th>Polymorphism bands (%)</th>
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<tbody>
<tr>
<td>UBC 815</td>
<td>(CT)$_3$ G</td>
<td>51.5</td>
<td>9</td>
<td>9</td>
<td>100</td>
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<tr>
<td>UBC 835</td>
<td>(AG)$_3$ YA</td>
<td>53.1</td>
<td>4</td>
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<td>100</td>
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<td>UBC 836</td>
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<td>Total</td>
<td></td>
<td></td>
<td>31</td>
<td>28</td>
<td>91.25</td>
</tr>
<tr>
<td>Average</td>
<td></td>
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<td>7.75</td>
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<td>91.25</td>
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</table>

ISSR = intersimple sequence repeat.

Discussion

Observation of male gamete development and pollination experiment. At present, lack of pollen is considered a trait related to apomixis in plants (Dong, 1987; Mao et al., 1995). During this study, the pollen development of ‘Mianli’ was observed, and the results showed that ‘Mianli’ experiences pollen abortion because of premature disintegration of the tapetum and abnormal meiosis of pollen mother cells, indicating that ‘Mianli’ is a strictly male-sterile cultivar. Many scholars have also found apomixis to be accompanied by male sterility in *Malus*. For example, Yang (1990) found this characteristic in *Malus xiaojinensis* and Lespinasse (1975) found this characteristic in *Malus hupehensis*. These results are consistent with our conclusion that ‘Mianli’ is male-sterile.

The fruiting pattern of plants can reflect the reproductive characteristics to a certain extent. Sexually reproducing plants cannot bear fruit if the stamens are removed. For facultative plants with a relatively low degree of apomixis, removing the stamens can significantly reduce the seed setting rate (Yao, 2005). The actual identification of apomixis is often performed by bagging and pollination experiments. For example, Fayeun et al. (2014) identified the facultative apomixis of five genotypes of fluted pumpkin (*Telfairia occidentalis* Hook. F.) by the emasculation and bagging method. Werpachowski et al. (2004) examined the apomixis and parthenocarpy of 22 species of *Asteraceae* in Curitiba, Paraná, southern Brazil through emasculation and bagging. Kissing et al. (2006) found evidence of apomixis in the invasive tussock grass *Nardus stricta* L. (*Poaceae*) in New Zealand through emasculation and pollination experiments. We designed four treatments, emasculation with bagging, no emasculation with bagging, artificial pollination, and natural pollination, for ‘Mianli’, and the results showed that under the treatments of emasculation with bagging and no emasculation with bagging, the seed setting rate was low and the number of seeds per fruit was very low, whereas the germination rate of this batch of seeds was 100%, which proved that ‘Mianli’ can produce some fertile seeds without pollination or fertilization. Therefore, ‘Mianli’ has the characteristics of a low degree of autonomous apomixis. Leblanc and Mazzucato (2001) and Van Dijk (2009) also believed that apomixis might exist if plants produce fertile seeds in the absence of pollen. In addition, compared with emasculation with bagging and no emasculation with bagging treatments, artificial pollination and natural pollination yielded a higher fruit setting rate and more seeds per fruit, indicating that the presence of pollen significantly improves the fruit setting rate and the number of seeds per fruit, and that

Fig. 5. Longitudinal sections of ovules showing apomictic embryo sac development of ‘Mianli’.

1. Multi-embryo sac development (6 d BFB).
2. Developing seed showing two embryo sacs (es), one is an apoporous binuclear embryo sac and the other is a mature embryo sac containing degenerated antipodals, fused polar nuclei (pn), and an elongated egg (ec) (1 d AFB).
3. Degenerated sexual embryo sac (3 d AFB).
4. A degenerated sexual embryo sac and an apoporous mononuclear embryo sac (2 d BFB).
5. Two proembryos and polar nuclei (10 d AFB).
6. The embryo sac has only proembryo. There is no endosperm development. (7) There are only primary endosperm nuclei (arrow). There is no proembryo in the embryo sac. (8) The egg cells and polar nuclei in the embryo sac develop independently to form spherical embryo and free-nuclear endosperm.

Four specific primers were used for amplification. The emasculation with bagging progeny of ‘Mianli’ had a high degree of uniformity. Figure 6 shows that the fruit-bearing progeny numbers 2, 6, 10, 11, 14, and 15 of ‘Mianli’ under emasculation with bagging treatment had band types that were completely consistent with those of the female parent for all four primers; therefore, they were truly apomictic progeny. In contrast, progeny 3, 5, 7, 12, and 13 had a specific band not found in the female parent, and one of the female parent’s unique bands was missing from 8 and 9. Twenty hybrid plants (artificial pollination) were detected by using the four primers (Fig. 7), and they had four band patterns: common bands of both parents, bands close to those of the female parent, bands close to those of the male parent, and missing common bands of both parents. During amplification using the four primers, no progeny with the same band pattern as the female parent appeared. Compared with the progeny under the treatment of emasculation, the genetic differences between the progeny under artificial pollination and the female parent were relatively large, and the uniformity of the progeny was relatively low. The combination of the amplification results with the four primers and the seed setting rate under artificial pollination showed that the progeny under artificial pollination were all sexually reproducing, and the seeds were from the fusion of the parents. Consistent with the results of artificial pollination, the amplification results of the progeny under natural pollination when using the four primers showed that no progeny had the same band types as the female parent (Fig. 8). The combination of the amplification results by the four primers and the seed setting rate under natural pollination showed that the progeny under natural pollination all came from sexual reproduction.
pollen has a significant role in fruit development. Whether the seeds of artificial pollination and natural pollination are the products of apomixis by pseudogamy still needs to be determined by genetic and molecular biological studies.

**Occurrence and development of sexual and asexual embryo sacs in 'Mianli'**. In most apomictic higher plants, the process of sexual reproduction usually ceases soon after the onset of apospory (Bicknell and Koltunow, 2004; Koltunow et al., 1998, 2000). Our observation of the embryo sac development of 'Mianli' showed the process of meiosis, the abortion process of some sexual reproduction at =2 d before blooming (Fig. 5-3 and 5-4), and the completion of some sexual reproduction. This phenomenon has also been reported by a study of *Malus hupehensis* by Liu et al. (2006). We also observed that in the few ovules under the treatment of emasculation with bagging, the apomictic embryo sacs began to develop after sexual and Koltunow reproduction failed (Fig. 5-4). Krahulcová et al. (2014), Neiman et al. (2014), and Tucker and Koltunow (2009) argued that apomixis and sexual reproduction can coexist in apomictic plants without mutual exclusion. When plants are not able to accomplish sexual reproduction, apomixis is likely to occur so that the species can continue, which is a manifestation of plant adaptation to changes (Wang et al., 2008). This phenomenon has also been found in aposporous *Pennisetum* and *Hieracium* species (Koltunow et al., 2000, 2011; Peel et al., 1997; Snyder et al., 1955), and in the study of *Malus hupehensis* by Liu et al. (2006). Generally, the development of a sexual embryo sac precedes development of an asexual embryo sac (Bicknell and Koltunow, 2004). This phenomenon was helpful for us to distinguish the sexual embryo sac from the aposporous embryo sac. We observed that when the sexual embryo sac had developed into a multinucleated embryo sac, the aposporous embryo sac was still in the stage of a binuclear embryo sac (Fig. 5-2).

The appearance time and frequency of the aposporous initial cells are related to the pathways of sexual reproduction and differ between species. For example, in paraffin sections of the pistil of *Malus hupehensis*, Liu et al. (2006) found that asexual embryo sac development starts near the flowering stage. Koltunow et al. (2011) discovered that somatic aposporous initial cells differentiated near meiotic cells in the *Hieracium subgenus Pilosella*. Zhang (2004) observed that the aposporous initial cells appear near the flowering stage. Our observation of the developmental process of the embryo sacs of 'Mianli' showed that one to four aposporous initial cells appear at each stage of ovule development. Most of the aposporous initial cells originate from the nucellar tissue cells or from the peripheral tissue cells. Therefore, the origins of aposporous initial cells of 'Mianli' have the characteristic of spatial multipositions (Figs. 4-2, 4-3, 4-4, 4-5, 4-6, and 5-4). This phenomenon is consistent with the observations of Hjelmqvist (1957) and Liao (1996) studying *Malus sieboldii* and *Malus tomentosa*. In addition, during the development of the embryo sacs of 'Mianli', many special nucellar cells develop into a small number of aposporous embryo sacs and proembryos, which is consistent with the known aposporous process, thus confirming that the apomixis of 'Mianli' belongs to the type of apomixis.

Under the treatment of natural pollination, ~4% of sexual embryo sac abortions were mainly attributable to insufficient fertilization. Because the endosperms are not divided, embryos in the ovule have no endosperms, resulting in the failure of embryo development. No division is initiated in the egg cells, and only endosperms without embryos develop during the late stage of ovule development, which is consistent with the result that an average of approximately six fertile seeds per fruit are obtained under natural pollination. After pollination is blocked (emasculation with bagging), not all egg cells and polar nuclei initiate division autonomously, and most embryo sacs are aborted because of the failure of spontaneous division of one of them, which is the main reason for the low seed setting rate of 'Mianli' in apomixis. The embryos of aposporous plants are formed by parthenogenesis of unreduced egg cells, but the endosperms of most plants require the fertilization of polar nuclei (i.e., pseudogamy). Only a few apomictic plants have been found, such as *Hieracium*, *Taraxacum*, and *Calamaegratis*, whose endosperms are formed by spontaneous division of the polar nucleus (i.e., autonomous apomixis) (Koltunow and Grossniklaus, 2003). In the ovary sections of 'Mianli' after emasculation with bagging, we saw that ~4% of the egg cells and polar nuclei in the embryo sacs divided autonomously to form embryos and free endosperm nuclei. These observations suggest that the apomictic endosperms of 'Mianli' can arise without fertilization and are formed by spontaneous division of polar nuclei, which is a rare type of autonomous apomixis.

**Apomixis and diploidy.** 'Mianli' is a male-sterile diploid that is capable of sexual reproduction and spontaneous aposporous apomixis under certain conditions. Gametophytic apomixis tends to occur most often in polyploids, and then at the tetraploid or greater levels (Asker and Jerling, 1992). However, in previous embryological studies, it was noticed that several sexual outcrossing (self-sterile) diploids of *Paspalum* occasionally develop an aposporous embryo sac beside the regular meiotic one (Quarin et al., 2001). Embryological observation of the diploid genotype Q3745 by Normann et al. (1989) revealed that between 8.3% and 26.8% of the ovaries included a meiotic and an aposporous-like embryo sac, suggesting some capability for apomictic reproduction. Re-examination of Q3754 ovaries by Siena et al. (2008) showed that 12.5% of them contained one sexual and one aposporous sac, confirming previous results.
Progeny tests were performed for two experimental families (H1 and S1) using heterozygous RAPD marker loci. The results presented indicate that 5 of the 95 plants from S1 showed the same heterozygous state as the mother plant for 14 RAPD loci, suggesting a clonal origin. Similar results were reported for Brachiaria (Naunova et al., 1999). The presence of aposporous embryo sacs in these plants suggests that the factors responsible for aposity are occasionally expressed in diploid plants. Mao et al. (1995) identified the reproductive capacity of 20 apple plants and found that the diploid Malus maer-
kangensis is male-sterile and that Malus zumi Mails is male-fertile; furthermore, both show a low fruit setting rate with a small number of plump seeds after emasculation with bagging. Therefore, it is suspected that diploid plants are capable of apomixis. These observations indicated that some ability of apomictic reproduction can be expected in the genus at the diploid level. These experimental results are consistent with the apomixis of the diploid ‘Mianli’, verifying the possibility of apomixis in diploid plants.

Identification of ‘Mianli’ progeny with ISSR molecular markers. With ISSR markers, nearly identical amplified bands were obtained for D4–10 × Poland single plant DNA and DNA derived from turfgrass started from seedlings on all gels, which again showed that D4–10 × Poland is highly apomictic (Goldman, 2013). We conducted DNA analyses on ‘Mianli’ progeny after emasculation with bagging using ISSR marker technology. The progeny populations were highly consistent at the molecular level, and the bands of several apomictic plants were completely consistent with those of the female parent. The other plants had slightly different bands, which may be attributable to the errors in the operation of emasculation with bagging or unknown variation; however, the reason for this will be investigated during another study. The present ISSR analysis showed that emas-
culation with bagging can produce plants with the same band types as the female parent, which again proves that ‘Mianli’ has the ability for apomixis under certain conditions and indicates that ‘Mianli’ faithfully transmits its genetic material to its apomictic progeny. At present, we have performed a preliminary study of apomictic characteristics of ‘Mianli’. The reasons for this phenomenon (the bands of several progeny of emasculation with bagging were slightly different from the female parent) and the evolutionary source of apomixis will be the subjects of our further experiments.

Qiao et al. (2010) identified the progeny of Loquat [Eriobotrya japonica (Thunb.) Lindl.] and believed that the hybrid progenies with a male parent-specific bands or their own specific bands were the true hybrid. This study used ISSR molecular markers to reveal that the progeny had one of four types of am-
progeny after emasculation or natural pollination: common bands of both parents; specific bands of the male parent; specific bands of the female parent; or their own specific bands that were not found in the male parent or female parent. None of the progeny bands was fully consistent with the bands of female parent. Therefore, it can be concluded that the progenies under artificial pollination or natural pollination are not the products of pseudogamy and are all true hybrids.

Conclusion

The results of this study showed that ‘Mianli’ is male-sterile diploid. ‘Mianli’ has the ability of apomixis, and its apomixis can occur without fertilization, but at a low inci-
dence. When ‘Mianli’ is stimulated by external pollen during the flowering period, it receives foreign pollen for sexual reproduction and produces sexual seeds. When there is no external pollen stimulation, to protect its own genetic stability, ‘Mianli’ initiates another reproductive mode, that is, apomixis, to produce apomictic progeny.

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