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Reproductive biology of Chinese herbaceous perennial Peony (*Paeonia lactiflora* Pall.) using the Paraffin Method

Biología reproductiva de la peonia herbácea perenne china (*Paeonia lactiflora* Pall.) usando el método de la parafina

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Abstract. Chinese herbaceous peony (*Paeonia lactiflora* Pall.) is a popular ornamental plant. However, little is known about its reproductive biology by histological analyses. Here, we used the paraffin wax method to study the reproductive biology of the Chinese herbaceous peony (*Paeonia lactiflora* 'Dafugui'). The results showed that the flower bud of Chinese herbaceous peony was a mixed bud. The course of flower bud differentiation included some developmental morphology periods, such as bract, calyx, petal, stamen, and pistil. The anther wall development was related to a dicotyledonous type. Pollen was mainly 2-celled, only few of them were 3-celled. Moreover, the mature pollen was circular with three germinal apertures. The number of pollen in petaloid anther was less than normal with different maturation periods. Superior ovary, polymerized follicle, anatropous ovule, polygonum type embryo sac, and serious embryo sac abortion were the features of the Chinese herbaceous peony found in this study. Moreover, in the Chinese herbaceous peony, the embryo was of a dicotyledonous type; the endosperm followed a nucleus model, and the seed coat showed double wall.

Keywords: Embryonic development; Petaloidy; Pistil; Stamen.

Resumen. La peonía herbácea china (*Paeonia lactiflora* Pall.) es una planta ornamental popular. Sin embargo, se conoce poco de su biología reproductiva por análisis histológico. En este estudio, usamos el método de la parafina para estudiar la biología reproductiva de dicha especie. Los resultados mostraron que la yema floral de la peonía (herbácea china) consistió de una yema mezclada, con más de un propósito morfológico. El curso de la diferenciación de la yema floral incluyó algunos períodos de la morfología del desarrollo: brácteas, cálix, pétalos, estamen y pistilos. El desarrollo de la pared de la antera estuvo relacionado al tipo de las dicotiledóneas. El polen principalmente fue de 2 celdas, solo unos pocos fueron de 3 celdas. El polen maduro fue circular con tres aperturas de germinación. El número de polen en la antera petaloide fue menor que lo normal con diferentes períodos de maduración. Un ovario superior, un folículo polimerizado, un óvulo anátropo, un saco embrionario tipo polígono, y serios abortos de sacos embrionarios fueron las características de la peonía herbácea China en este estudio. Más aún, el embrión en la peonía fue del tipo de las dicotiledóneas; el endosperma siguió un modelo núcleo, y la cobertura de la semilla mostró una pared doble.

Palabras clave: Desarrollo del embrión; Petaloide; Pistilo; Estamen.

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INTRODUCTION

Paeonia lactiflora is a branch of *Paeoniaceae* pertaining to *Paeonia L.*, and has various common names such as Jiangli, Chuoyue, Lanweichun, Dianchun, Meiguhua, Liuyi and so forth in China (Ji et al., 2012; Shen et al., 2012). It is a traditional flowering and ornamental plant, which is found abundantly in China, and is well known to be one of the world's oldest cultivated flowers. For a long time, Chinese herbaceous peony was extremely popular and had equal attention with tree peony species. In addition to its ornamental value, Chinese herbaceous peony was reported because of the medicinal effects such as anti-aging, analgesic, blood activation and stasis dissipation as well as hidroschesis and menstruation regulator (Shu et al., 2012; Schmitzer et al., 2013).

Plant reproductive biology was focused on the plant developmental process, and a meticulous understanding of this is needed for plant improvement, whether by conventional or biotechnological methods. There is a large number of reports on reproductive biology using the embedding paraffin method in different species popular in China such as *Castanea mollissimma* (Liu et al., 2012), *Pyrus* sp. L. (Yuan et al., 2007), and *Sargentodoca cuneata* (Wang et al., 2007). Additionally, the process of sexual reproduction in many plants have been researched more in-depth (Zhou et al., 2011; Zou et al., 2012).

At this moment, Chinese herbaceous peony's research on its reproductive biology is mainly focused on the flower bud differentiation (Mo et al., 2008), microsporangium development (Kong et al., 2004), fertilization and embryo development (Yaping, 2013) which provide the theoretical basis or foundation for its taxonomical study. This article aimed to study the Chinese herbaceous peony's reproductive biology using the paraffin method, which provided a theoretical basis for its development process research and regulation of reproduction. On the other hand, this work would establish a suitable media to lay a foundation for further studies on developmental biology and reproductive physiology.

MATERIALS AND METHODS

Chinese herbaceous peony (*Paeonia lactiflora* 'Dafugui') was collected in the Heilongjiang Forest Botanical Garden, China (Fig.1-A). Bud, stamen, pistil, petal, and seeds were chosen at regular intervals (every 3 days) for fixation since September of the previous year, when flower bud differentiation began. All samples were preserved and held in formalin-acetic acid-alcohol (FAA) (per 100 mL: 5 mL formalin, 5 mL glacial acetic acid, 35 mL distilled water and 50 mL ethanol) at room temperature (O'Brien & McCully, 1981).

The samples were preserved in FAA following embedding, sectioning and staining of the tissue. In the process of paraffin embedding and microtome sectioning, whole tissue were taken out from the FAA followed by rinsing 2 to 3 times with distilled water for 3 min. Longitudinal or cross sections 3 to 4 μm thick were cut from each tissue. It depended on tissue type, for example, tissues for flower bud research were sliced in lengthways while other tissues in breadthwise. The tissue discs were stored again in 70% ethanol until the paraffin embedding procedure began. The discs were sequentially dehydrated in a graded alcohol series (70%, 85%, 95% and 100% ethanol, respectively, for 1 h except for that of 95% which took 24 h) and cleared with xylene for 2 h, embedded in paraffin wax, and sectioned at 5 to 10 μm on a rotary microtome (Leica CM 1850). At last, ultra-thin sections (3 to 4 μm) were photographed on a light microscope (Olympus BH-2-C5060WZ) after stained with safranin and fast green.

RESULTS

The differentiation of flower bud. The bud of the Chinese herbaceous peony is a mixed one. At the beginning of September, under proper nutrition and flowering hormonal conditions, the bud primordium enters into a flower bud differentiation phase, and from vegetative growth to the reproductive phase. Early September to late September is the time for the bract primordium differentiation. The sepal primordium starts to develop from late September to middle October. Petal primordia start to shape from mid-October to late November. From mid-November to early March of the following year, stamen primordia emerge, and develop. In April, pistil primordium starts to form. The bud time is from early to the middle of May.

Transformation from vegetative growth to reproductive growth. During vegetative growth period, the shoot apex is dome shaped as demonstrated in the Fig. 1-B, and generates constantly new leaf primordia. During the reproductive growth period the growing area of the shoot turns to flat (Fig. 1-C).

The bract primordium formation. When the reproductive growth period starts, the prominence of the bract primordium is noticeable (Fig. 1-D).

The period of sepal primordium. At the end of the later stage of bract primordium, sepal primordia start to form (Fig. 1-E). After the sepal primordium stage, then it encloses bloom entirely.

The period of petal primordium. The topmost growing point unfolds and expands greatly, which then turns into a cupular-shaped flower tray (Fig. 1-F). Interestingly, the sepal primordium having concentric circles is at the edge of the flower tray. Petals, which are at the edge of the flower tray, are called asexual petals. As shown in the Fig. 1-L, the shapes of these asexual petals appeared to have a rift. In most of species, the phase of petal primordium completes within the period from November to March of next year.

The period of stamen primordium. In late March, stamen primordium forms inside sepal primordium, and arranges

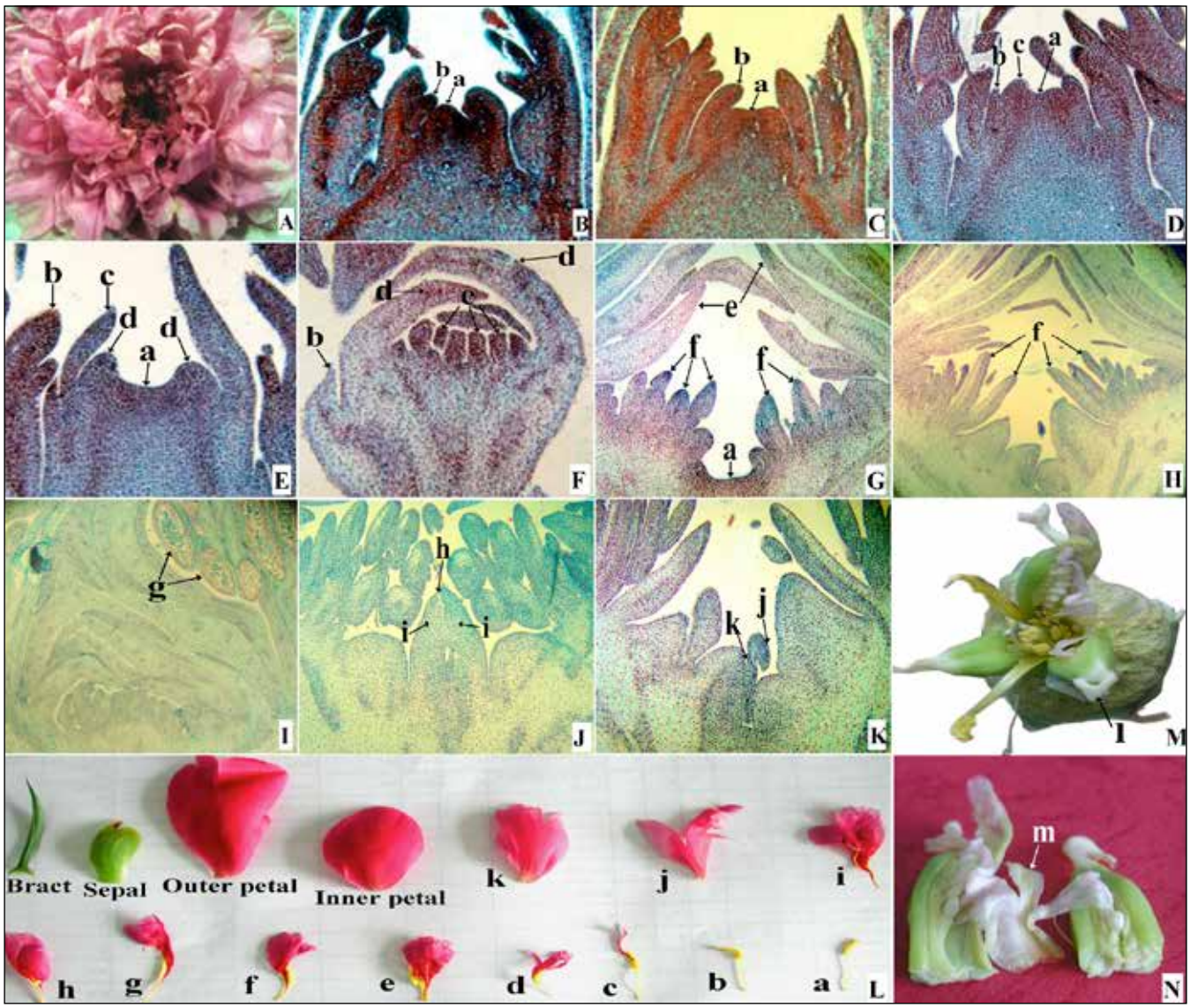

Fig. 1. The process of flower bud differentiation. (A) "Dafugui" flower; (B) Growth cone in Vegetative growth (100×); (C) Growth cone in Reproductive stage (100×); (D) Bract Primordium (100×); (E) Sepal Primordium (100×); (F) Petal Primordium (100×); (G) Stamen Primordium (100×); (H) Later stage of stamen growth (100×); (I) Petaloid anther (100×); (J) Carpel union (100×); (K) Stigma shows up on stamen (100×); (L) the process of petaloid anther; (M) Partial petaloid pistil; (N) Two petaloid pistils linked together.
(a) growing point; (b) leaf prinordium; (c) bract primordium; (d) sepal primordium; (e) petal; (I) petaloid pistil; (m) petaloid pistil connected.
Fig. 1. Proceso de diferenciación de la yema floral. (A) flor "Dafugui"; (B) Cono de crecimiento durante Crecimiento Vegetativo (100×); (C) Cono de crecimiento en estado reproductivo (100×); (D) Primordio de bráctea (100×); (E) Primordio de sépalos (100×); (F) Primordio de pétalos (100×); (G) Primordio de estamen (100×); (H) Estado tardío de crecimiento del estamen (100×); (I) Antera petaloide (100×); (J) Unión de carpelos (100×); (K) Estigma que se muestra sobre el estamen (100×); (L) Proceso de antera petaloide; (M) Pistilo parcialmente petaloide; (N) Dos pistilos petaloides unidos.
(a) Punto de crecimiento; (b) primordio de hoja; (c) primordio de bráctea; (d) primordio de sépalo; (e) pétalo; (I) pistilo petaloide; (m) pistilo petaloide conectado.

itself in concentric circles (Fig. 1-G). However, with the growth of stamen primordium, the circular shapes change to cylindrical, and the central part grows faster than that of the surroundings (Fig. 1-H). The petal primordium is not the petaloidy, its top differentiates as anthers while the lower part become filaments. The petals from petaloidy of stamen and pistil are called sexual petals, which have sign of stamens (yellow) (Fig. 1-L).

The period of pistil primordium. As shown in Figure 1-J, with increasing in the petaloidy of the stamens, ventral suture

is produced at pistil primordium. Then stigma and style begin to divide, as shown in Figure 1-J.

At first, the petaloidy of stamen primordium is dehisced along ventral suture, then expands, and finally turn into a petal. In few cases, the pistil just generates into a partly petaloidy (Fig. 1-M), while few of them even bond together as shown in Figure 1-N.

Microsporogenesis and development of the male gametophyte

The formation of anther wall. As shown in Figure 2-A, it is observed that the anther primordial is subrotund from the transverse section. Its outer surface has a circular layer of small cells, which arrange tightly. Due to rapid fission of the corner cell, it turns more protruding, and results in the anther to form a four valve-shape as depicted in Figure 2-B. Then within the skin of each valve cell, sporogonium starts to differentiate. Generally, the archesporial cells have bigger volume, denser cytoplasm, more noticeable nucleus, and more irregular polygons than other cells. The anther wall has two layers of cells. The primary wall cells form the outer layer, and the primary sporogenous cells are located in the inner layer. Then primary wall cells continue the periclinal division, form the inside and outside layers of the secondary wall cells. The outside cells undergo the periclinal division, and form the outer (closed to the inner layer of epidermis) endothecium and the inner midlayer. The inside cells directly develop into a petal layer.

The development of Chinese herbaceous peony anther wall belongs to a dicotyledonous type. After it differentiates completely, the anther wall comprises the epidermis, fibrous endothecium, one middle layer, and tapetum, each of them has one epidermal cell as shown in Figure 2-C.

The transformation of anther wall during the anther development. The development of anther wall takes different changes. Firstly, the epidermal cell is a part of the isodiametric cell. Then it undergoes the anticlinal division to increase the number of cells to fit the rapid growth of the internal organization. As shown in Figure 2-D, the anther becomes flat step by step during its growth. As shown in Figure 2-E, up on the mature anther, the cells still exists in the outermost layer and continues the regulation.

The endothecal endothecium has multiple layers of cells giving it a fibrous nature. Cell walls form various arcus from the inner tangential wall in the outward and upward directions, while there is nothing at the junction of two pollen sacs. As depicted in Figures 2-C, 2-D, the endothecium cells transform slowly from the flat shape to approximately a circular shape. When anther enters the maturation period, its development reaches the highest degree. As shown in Figures 2-E and 2-F, up on the mature anther, cells get rearranged in a fence-like arrangement. After maturation, anther begins to dehiscence. In Figure 2-F, the anther cracks at the joint of two pollen sacs and releases the pollen grains from those cracks.

The middle lamella which has one layer of cells is situated in the inner side of endothecal endothecium. As shown in Figures 2-C and 2-D, during the developing stage of microspore mother cell, middle lamella flattened and then gradually disintegrates. A middle lamella is devoid in mature anther.

The tapetum is one of the most inner layers of the anther wall. It is a homotypic cell layer derived from the primary wall cells. During the anther development process, the tapetum cells are always in their own original position. The tapetum secretes various kinds of substances on the surface to provide the nutrition for the microspore. However, in the mature pollen, the tapetal cells disappear entirely as depicted in Figures 2-E, 2-F.

The phase of microsporogenesis. The primary sporogenous cells, resulted from the archesporium cell division, develop into secondary sporogenous cells which are called microspore mother cell by mitosis. Each microsporocyte undergoes the meiotic division into tetrad haploid which is called a microspore (Fig. 2-G, 2-H).

Development of the male gametophyte. The microspore tetrad is surrounded by the callose wall which gradually disappears (Fig. 2-G). After a short period, the microspore gets much bigger than it was before as demonstrated in Figure 2-H. With increasing size, microspore nucleus gradually moves to the side from center as shown in Figure 2-I. At this time, microspore becomes roundness or oval. Microspores, uninucleate, further mitosed into two daughter nucleus, one is generative nucleus, close to pollen wall, the other one is vegetative nucleus, towards vacuole (Fig. 2-J). Generally, the generative cell is small and in convex shape with less cytoplasm. On the other hand, the vegetative cell is larger as it contains the large vacuole and most of the cytoplasm of the original microspore.

As shown in Figure 2-K, when the anther gets mature, the pollen grains have 2-cells ahead of powder which is dispersed in time; only a few of them are 3-celled as shown in Figure 2-L. Some scholars think that the original species were 2-celled. So it can be concluded that the Chinese herbaceous peony is a transition plant from initial species to an evolutionary species. The ripe pollens of Chinese herbaceous peony are round with three germination apertures as depicted in Figure 2-K.

The microsporogenesis and male gametophyte development of petaloid stamen

The formation of anther wall. There are usually two or more stamens which merge together before petaloidy, which can be known according to the number of vascular bundles in petaloid stamen as shown in Figure 3-A. There exist three vascular bundles in anther before its petaloidy. In most cases, there is only one vascular bundle in it. There is no difference between anther wall and normal ones in their development.

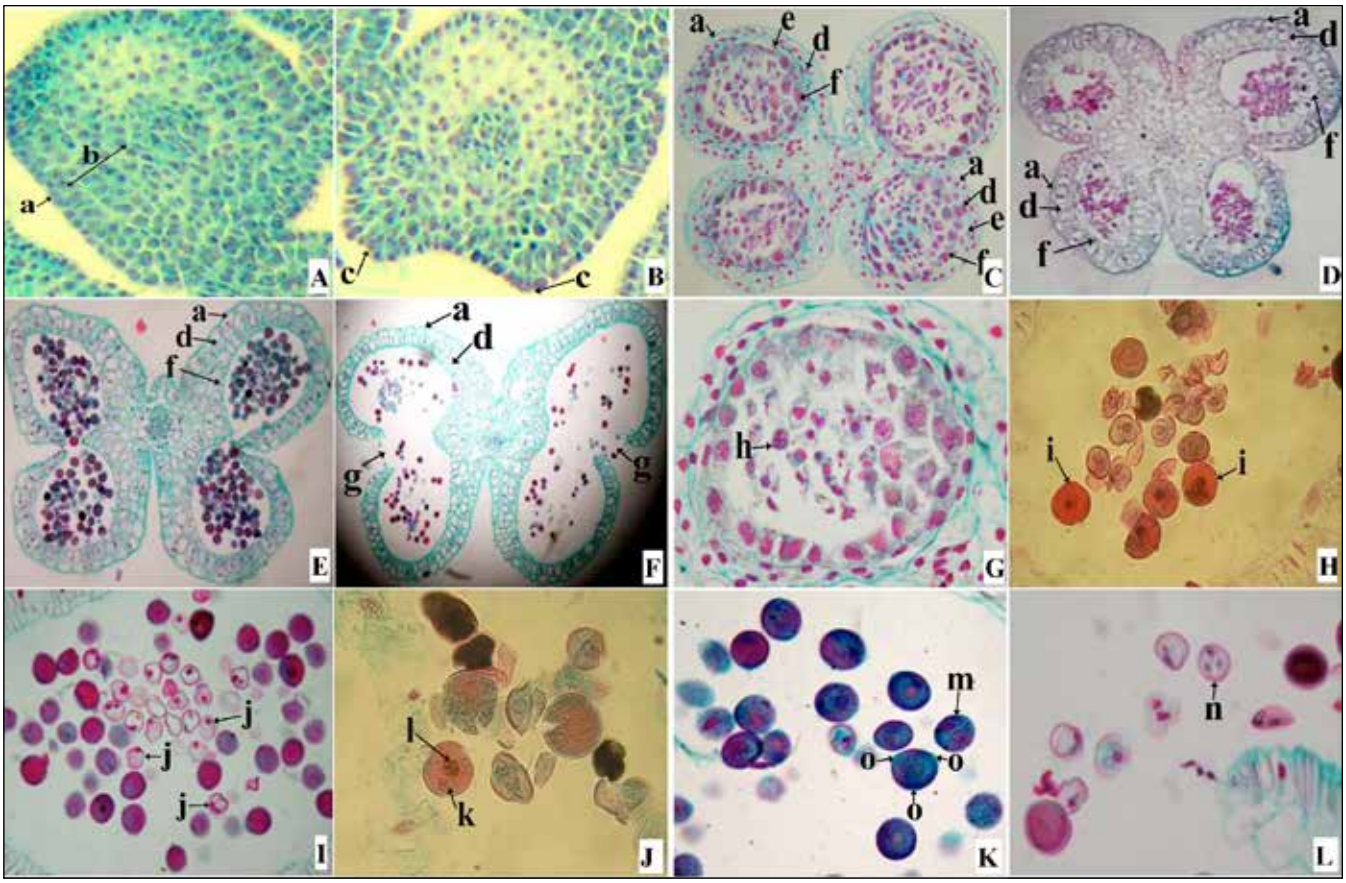

Fig. 2. The process of microsporogenesis and development of male gametophyte. (A) Anther Primordium (100×); (B) Sporogonium division (100×); (C) Four layer wall of anther (100×); (D) Tapetum cell transfiguration (100×); (E) Mature anther (100×); (F) Anther cell powder dispersion (100×); (G) Tetrad (400×); (H) Mononuclear pollen (400×); (I) Pollen of Mononuclear one-side stage (400×); (J) 2-nucleu pollen grain (400×); (K) Mature 2-nucleu pollen grain (400×); (L) 3-cell pollen grain (400×).
(a) epidermis cell; (b) endothelial cell; (c) the corner of sporogonium; (d) endotheca endothecium; (e) middle lamella cell; (f) tapetum cell; (g) anther crack; (h) microspore tetrad; (i) microspore; (j) microspore with one-side nucleus; (k) generative nucleus; (l) vegetative nucleus; (m) 2-cell pollen grain; (n) 3-cell pollen grain; (o), germinal aperture.

Fig. 2. Proceso de microsporogénesis y desarrollo del gametófito masculino. (A) Primordio de antera (100×); (B) División del esporangio (100×); (C) Pared de cuatro capas de la antera (100×); (D) Transfiguración de la célula Tapete (100×); (E) Antera madura (100×); (F) Dispersión del polvo de la célula de la antera (100×); (G) Tetrad (400×); (H) Polen mononuclear (400×); (I) Polen de un lado mononuclear (400×); (J) Grano de polen de 2 núcleos (400×); (K) Grano de polen maduro de 2 núcleos (400×); (L) Grano de polen de 3 células (400×).
(a) célula de la epidermis; (b) célula del endotelio; (c) la esquina de un esporangio; (d) endotecia endoteca; (e) célula de la lamela media; (f) célula del tapete; (g) rajadura de la antera; (h) tétrada de microspora; (i) microspora; (j) microspora con núcleo de un lado; (k) núcleo generativo; (l) núcleo vegetativo; (m) grano de polen de 2 células; (n) grano de polen de 3 células; (o), apertura germinal.

Changes in the anther wall. Along with anther growth, cells within each layer of petaloid stamen anther wall may have different changes such as the development of epidermis cells, thickened fibrous layer, disappearance of middle lamella and tapetum, which are almost the same as that of normal stamen (Fig. 3-B, 3-G). But petaloid stamen has external form changes which are quite different from those of normal stamen.

As shown in Figure 3-B, two or more stamen would merge together. Besides, the anther cell shows a tendency to sink inwards. First, each anther cell tends to sink inward, then it shrunken obviously, showing a sheet structure. As for the other anther cells, cell wall begins the same situation and connects to the other sides of the wall, which comes to more than 4 anther cells. As shrinking continues, sheet structure stretches as shown in Figures 3-D, 3-E, 3-F. Finally, anther cells probably become sheet structure completely (Fig. 3-G).

Microsporogenesis. As for the microsporogenesis in stamens, they have the same process, no matter petaloid one or normal one.

Development of the male gametophyte. The development of male gametophyte within petaloid stamen is almost the same

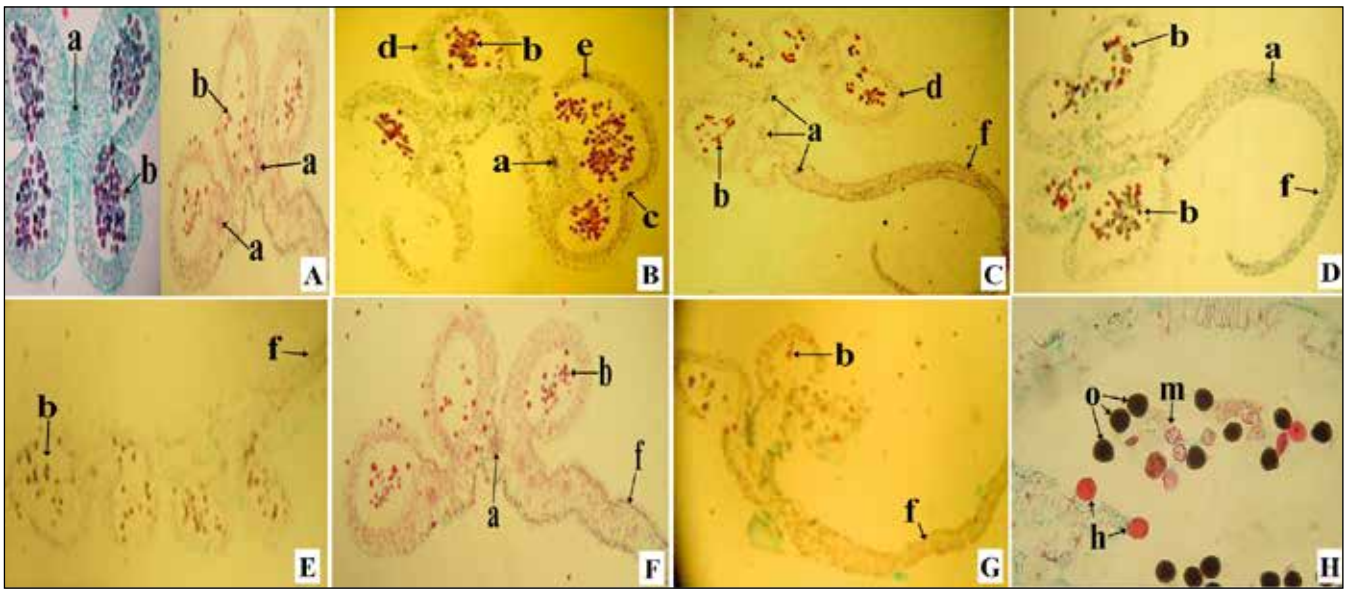

Fig. 3. The process of microsporogenesis and development of male gametophyte in petaloid stamen. (A) The contrast of normal stamen and petaloid stamen (100×); (B) A few anther union; (C) Petaloid petal emerges (100×); (D~F) Petaloiding anther cell (100×); (G) Petaloid anther dehisce (100×); (H), Dispar mature period of pollen (400×).
(a) vascular bundle; (b) pollen; (c) depression of anther cell; (d) anther cell; (e) intine of anther cell; (f) laminated-like structure; (g) mature pollen; (h) immature pollen.

Fig. 3. Proceso de microsporogénesis y desarrollo del gametófito masculino en el estamen petaloide. (A) Contraste del estamen normal y el estamen petaloide (100×); (B) Algunas uniones de anteras; (C) El pétalo petaloide emerge (100×); (D~F) Célula de la antera petaloide (100×); (G) Apertura en grietas de la antera petaloide (100×); (H) Período diferente de maduración de polen (400×).
(a) haz vascular; (b) polen; (c) hundimiento de la célula de la antera; (d) célula de la antera; (e) intina de la célula de la antera; (f) estructura semejante a una lámina; (g) polen maduro; (h) polen inmaduro.

as that of the normal stamen. It undergoes the process from period that mononuclear tends to one side, dicaryophase, and pollen mature as well. In addition, the external form of mature pollen is the same as that of a normal one as depicted in Figure 3-B. But the difference is that the number of pollen in petaloid stamen is significantly less than that in a normal stamen as shown in Figure 3-A.

Development of the megasporogenesis and female gametophyte

Development of the ovule and megasporogenesis. As depicted in Figure 1-M, Chinese herbaceous peony has a superior ovary which contains three or more carpels. Each carpel forms a locule with many ovules (Fig. 4-A, 4-B). Figure 4-C, 4-D, and 4-E demonstrate that, the placentation is marginal, containing the ventral and dorsal sutures with the ovule being anatropous.

During locule forms, part of cells at the basal placenta are in a periclinal division into a small enation which then increases rapidly and transforms into an ovule primordium. The ovule primordium develops further; the anterior end develops into a nucellus, while the base forms a funicle attached to placenta.

With the further growth of ovule, there forms protrusion around, becoming integument primordium. In the meantime, a cell under the topmost epidermis of the nucellus amplifies and develops into sporogonium. As shown in Figure 4-F, the ovule is tenuinucellate and its integument develops rapidly. The ovule has two layers of integument, (1) the inner layer of which it forms, and (2) the outer layer. The nucellus is completely surrounded by the integument upwards, leaving a small pore called as micropyle. Interestingly, the endostome and exostome show zigzag rather than a straight line as shown in Figure 4-F. As depicted in Figure 4-E, the ovule is very close to the funicle, so the Chinese herbaceous peony is somehow an anatropous ovule.

Development of the female gametophyte. After meiosis, megasporocytes form a megaspore tetrad, out of which, there is only one megaspore at the chalazal end which develops into a functional one, while the rest of three megaspores retrograde. In order to further develop into mononuclear, the volume of the functional megaspore increases continuously (Fig. 4-G). In its first division, the functional megaspore is separated into two nucleuses without wall, known as 2-nucleate embryo sacs as depicted in Figure 4-H. These 2-nucleate embryo sacs separate into 4-nucleate, and 8-nucleate embryo sacs further. Then, the 8-nucleate embryo sacs continue developing; the two polar nucleuses move close together towards the center of the embryo sacs as shown in Figure 4-I. Finally, these two polar nucleuses mix to form a diploid nucleus, known as a secondary nucleus

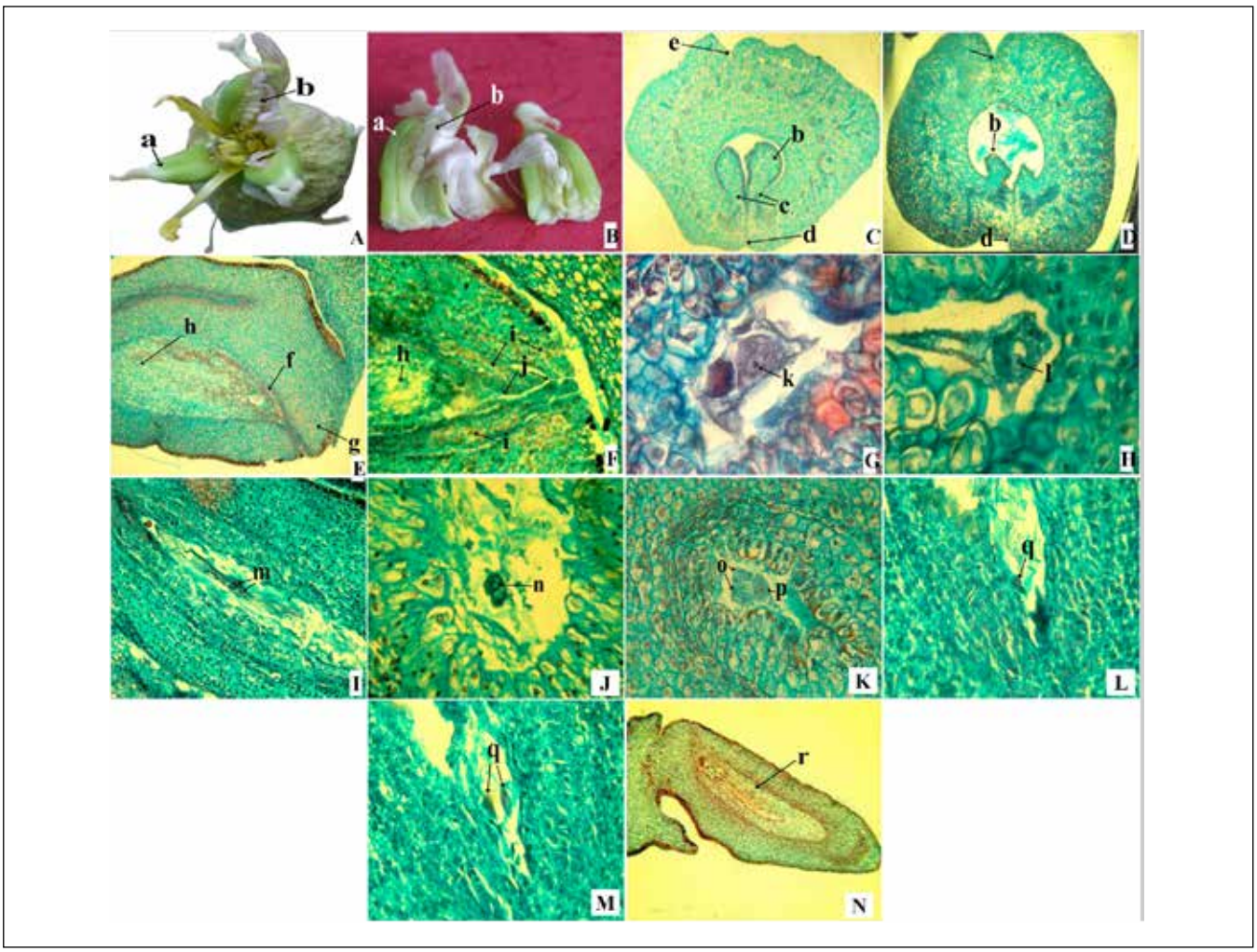

Fig. 4. The process of megasporogenesis and female gametophyte. (A) Partial petaloid pistil; (B) Two petaloid pistils link together; (C) Marginal placentation (40×); (D) Ventral suture and dorsoventral suture (40×); (E) Anatropous ovule (40×); (F) Micropyle (100×); (G) Mononuclear embryo sac (400×); (H) Dicaryotic embryo sac (400×); (I) Primary polar nucleus (400×); (J) Secondary polar nucleus (400×); (K) Egg apparatus (400×); (L~M) Antipodal cell (400×); (N) Abortive embryo sac (400×).
(a) carpel; (b) ovule; (c) placenta; (d) dorsoventral suture; (e) ventral suture; (f) anatropous ovule; (g) funicle; (h) nucellus; (i) integument; (j) micropyle; (k) mononuclear embryo sac; (l) embryo sac with two polar nuclei; (m) polar nucleus; (n) secondary nucleus; (o) synergid; (p) egg cell; (q) antipodal cell; (r) abortive embryo sac.

Fig. 4. El proceso de megasporogénesis y gametofito femenino. (A) Pistilo petaloide parcial; (B) Dos pistilos petaloides unidos; (C) Placentación marginal (40×); (D) Suturas ventral y dorsoventral (40×); (E) Ovulo anátropo (40×); (F) Micrófilo (100×); (G) Saco embrionario mononuclear (400×); (H) Saco embrionario dicariota (400×); (I) Núcleo polar primario (400×); (J) Núcleo polar secundario (400×); (K) Aparato de huevos (400×); (L~M) Célula antípoda (400×); (N) Saco embrionario abortivo (400×).
(a) carpelo; (b) óvulo; (c) placenta; (d) sutura dorsoventral; (e) sutura ventral; (f) óvulo anátropo; (g) funículo; (h) nucello; (i) integumento; (j) micrófilo; (k) saco embrionario mononuclear; (l) saco embrionario con dos núcleos polares; (m) núcleo polar; (n) núcleo secundario; (o) sinergia; (p) célula huevo; (q) célula antípoda; (r) saco embrionario abortivo.

(Fig. 4-J). The two regular-shaped synergids of micropylar end and pear-shaped egg cell show a triangular arrangement as depicted in Figure 4-K. The chalazal end cells are called as the antipodal cell (Fig. 4-L, 4-M). Based on the development process above, it can be concluded that, the formation of the Chinese herbaceous peony embryo sacs belongs to a polygonum type. The embryo sac gradually expands along with the cell nucleus division, especially towards a long axis direction.

Embryo sac sterility. During the development process of the Chinese herbaceous peony embryo sac, sterility of the embryo sac can be observed as shown in the Fig. 4-N. Shrinkage of the embryo sac might begin after the formation of the integument and the 8-nucleate embryo sac.

Development of embryo and endosperm. When double fertilization finishes, the embryo and endosperm development

start. There is no distinct suspensor during the embryonic development. The original embryo is just a mass of cells (Fig. 5-A). *Paeonia lactiflora* has a nuclear-endosperm. The division of the primary endosperm nucleus is not associated with the formation of cell wall which just occurs in between dissociative nucleus in late development stage, then cell formats. The nuclear endosperm still exists if it is around the embryo, and that far away from embryo becomes cellular type (Fig. 5-A~5-G).

Once original embryo divides into almost fifty cells, the dermatogen can be discernable. Then it comes to a globular embryoid period as shown in Figure 5-B. After the increased volume of the globular embryo, further divided cells spread the surrounding area. This distributional change indicates the beginning of the formation of the heart-shaped embryo as shown in Figure 5-C. This situation is more obvious in the heart-shaped embryo development stage. Thanks to inconsistent cell division and expansion, in the hypocotyl region, embryo begins to elongate into a torpedo shape as depicted in Figure 5-D. In the metaphase of torpedo-shape embryo, along the vertical axis of embryo, mitosis turns it into a Y shaped distribution graph as depicted in Figure 5-E. The Fig. 5-F demonstrates that the peripheral part of embryo starts to divide again, resulting in the mature embryo which lies near the micropyle.

Polyembryony happens during the embryonic development of the Chinese herbaceous peony "Dafugui" (Fig. 5-G). It is produced within the same embryo sac.

Development of seed coat. As shown in Figure 4-F, the Chinese herbaceous peony has two layers of integument. More cells layers of the inner integument locates near the chalazal end. This contrasts to the micropylar end which only has 3 or 4 layers. The outer integument cell layer is obviously more than that of the inner integument, and is divided into two kinds. The epidermal cells have a near rectangular shape and are tightly arranged lengthways. Inside the epidermis, there are many layers of parenchymal cells, and the shape of cells becomes from close to equal diameter to irregular. However, they are not as tight as epidermal cells.

During embryonic development, Figure 6 depicts the increased fibrosis of the epidermis cells of the primine which elongate lengthways and arranges tightly.

For outer integument, the cytoplasm close to the epidermal cell reduces slowly and cells shrink as well. Cells in the fibrovascular tissue also become smaller. Finally, inside the epidermal cells, a layer of palisade tissue cells are differentiated. The inner integument cell extends transverse, and the cells become flatter as they get closer to outer integument. It can be concluded that the Chinese herbaceous peony has two layers of seed coats, exotesta and endopleura. The exotesta is generated by the development of the outer integument, and contains one layer of epidermis cell and one layer of palisade cells. The endopleura stems from the development of the inner integument with more cell layers side-to-side setup.

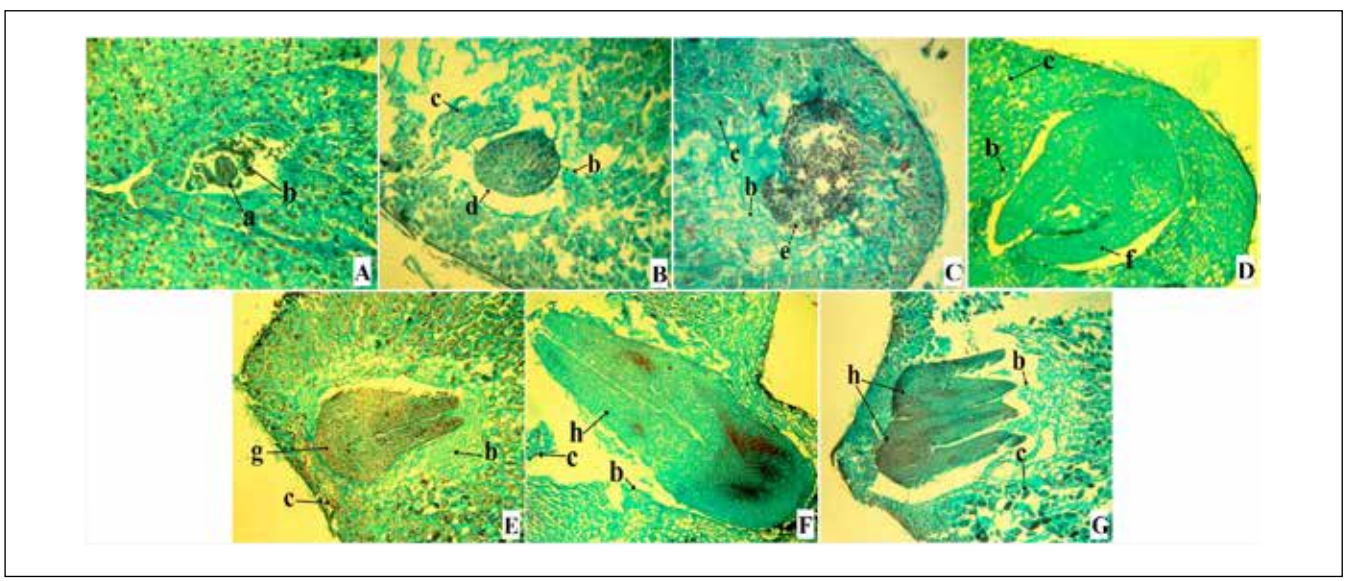

Fig. 5. The development of embryo and endosperm. (A) Proembryo period (100×); (B) Ball-shaped embryo (40×); (C) Heart-shaped embryo (40×); (D) Torpedo-shaped embryo (40×); (E) Cotyledon (40×); (F) Mature embryo (40×); (G) Polyembryogeny (40×).
(a) proembryo; (b) nuclear type endosperm; (c) cellular type endosperm; (d) globular embryo; (e) heart-shape embryo; (f) torpedo-shape embryo; (g) early cotyledon somatic embryo; (h) mature cotyledon somatic embryo.

Fig. 5. Desarrollo del embrión y el endosperma; (A) Período pre-embrionario (100×); (B) Embrión en forma de campana (40×); (C) Embrión en forma de corazón (40×); (D) Embrión en forma de torpedo (40×); (E) Cotiledón (40×); (F) Embrión maduro (40×); (G) Poliembriogenia (40×).
(a) pre-embrión; (b) endosperma de tipo nuclear; (c) endosperma de tipo celular; (d) embrión globular; (e) embrión en forma de corazón; (f) embrión en forma de torpedo; (g) cotiledón temprano del embrión somático; (h) cotiledón maduro del embrión somático.

DISCUSSION

As to *Paeonia lactiflora*, studies on flower bud differentiation are mature and comprehensive (Wang et al., 1991; Lv et al., 2009). The study of microspore growth focused on the volume of pollen and its external form, while study on megaspore' growth focused on that from nucleus development to four nucleated embryo stages (Kong et al., 2004). There are many studies on the macroscopic features of petalody core of both male and female ones (Kun, 2008) but few done by microscope, and no reports have been found about double fertilization. For embryonic development, there is only one study on early development of embryo and endosperm (Peng, 2000), but no post-embryonic development reports. Also there is a characterization research about seed coat (Sun et al., 2012) but none of its development process. So our work filled these knowledgement gaps and we hope these results would benefit for further breeding and propagation of the Chinese herbaceous peony.

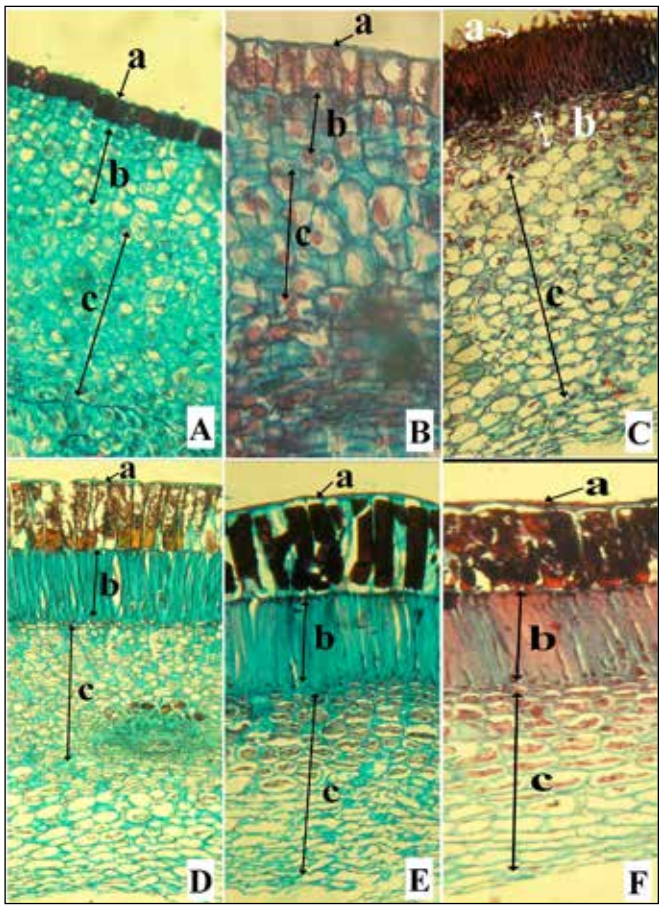

Fig. 6. The development of seed coat. (A) seed coat at mononuclear embryo sac stage (1000×); (B) seed coat at mature embryo sac stage (1000×); (C) seed coat at ball-shaped embryo stage (1000×); (D) seed coat at heart-shaped embryo stage (1000×); (E) seed coat at torpedo-shaped embryo stage (1000×); (F) seed coat at cotyledon embryo stage (1000×).
(a) epidermis cell of primine; (b) parenchyma cell of primine; (c) secundine.

Fig. 6. Desarrollo de la cobertura de la semilla. (A) Cobertura de la semilla en el estado de saco embrionario mononuclear (1000×); (B) Cobertura de la semilla en el estado de saco embrionario maduro (1000×); (C) Cobertura de la semilla en el estado embrionario en forma de campana (1000×); (D) Cobertura de la semilla en el estado del embrión con forma de corazón (1000×); (E) Cobertura de la semilla en el estado de embrión en forma de torpedo (1000×); (F) Cobertura de la semilla en el estado embrionario de cotiledón (1000×).
(a) célula epidérmica de la primina; (b) célula del parénquima de la primina; (c) secundina.

There are major possible causes of abortion as follows: there may happen serious stamen or pistil petaloidy in some of *Paeonia* sp., which results in a noticeable reduction of the number of males, i.e. a significant reduction of anthers (Zhou Yiling, 2011). However, the exact reason and mechanism of petoloidy stamens and pistils still remains unknown. The current data demonstrated that the petalody is closely associated with the nutritional conditions and genetics (Zheng et al., 2003). Interestingly, the petalody finishes inside the buds. If the genetic code of stamen or pistil is interrupted or damaged, planation happens on top of the cylindrical stamen primordium, and elongates into petals. The petalody of stamen primordium is the main source of the increased petals. According to the observation, Wang thinks that petaloidy of stamen or pistil shows both genetic individuality and integrity, or locality (Wang, 1991).

During our experiment, we found that there was two or more stamen merged together before they turn into a petaloidy one. And before petalody, three vascular bundles were observed in stamen anther. However, only one vascular bundle should be found in consistence with Chen's study result (Chen et al., 2007). Anther wall's growing process is almost the same than that in the normal anther.

Secondly, after petalody, for some stamen, it is found that male gametophyte in petaloid stamen has the same growing process as that of normal ones. They also undergo the phase of mononuclear towards the side, dicaryophase and matured pollen at last (Kong et al., 2002). But the amount of miospore and microspore within petaloid stamen anthers is lower than that in normal anthers (less than 1/3) and with different maturity as seen in Figures 3-H, 3-I.

Thirdly, embryo sac decreased or vanished after petaloidy in stamen, resulting in egg declining in number, as seen in Figures 4-A, 4-B and 4-N.

Some researchers observed that the seed coat of Chinese herbaceous peony is considered to be ariled and resulted from placenta growing protuberantly as mentioned in the earlier report (Sun et al., 2012). However, in this article, we showed that the seed coat of the Chinese herbaceous peony is real one with two layers (exotesta and endopleura). Exotesta is generated from integument, including inside and outside parts (i.e., one layer of epidermis cell and one layer of paliform cell). Endopleura is derived from inner integument, with multilayers and arranged crosswise.

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