Research paper

Sporogenesis and Gametophyte Development in the Chinese Chestnut (*Castanea mollissima* Blume.)

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【Summary】

Chinese chestnut (*Castanea mollissima* Blume.) is a widely distributed fruit tree and well known for its ecological and economic value. In order to evaluate obstacles to sexual reproduction in *C. mollissima*, microsporogenesis, megasporogenesis, and development of the male and female gametophytes of the *C. mollissima* 'Yanshanzaofeng' cultivar were examined by a paraffin section technique and scanning electron microscopy. Results showed that the anther wall was of the basic type and consisted of an epidermis, endothecium, middle layers, and tapetum. The tapetum was of the glandular type. Cytokinesis during meiosis of the microspore mother cell was of the simultaneous type, tetrads were tetrahedral, pollen grains were the 2-cell type, and development of the embryo sac was of the *Polygonum* type. In total, 16~18 ovules were found in the ovary axis, and the ovules were anatropous, bitegmic, and crassinucellate. Before fertilization, the 2 polar nuclei fused into a secondary nucleus. The mature embryo sac was made up of 7 cells. Abnormal embryo sacs or abortive ovules were observed in the ovary. The abortive ovules are likely the cause of the low seed set in *C. mollissima* 'Yanshanzaofeng'.

Key words: *Castanea mollissima*, megasporogenesis, microsporogenesis, male gametophyte, female gametophyte.

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研究報告

板栗大小孢子的發生和雌雄配子體的發育

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摘 要

中國板栗是廣泛分布的果樹，其具有生態和經濟價值。為了探討其發育過程中是否存在生殖障礙，利用石蠟切片和掃描電鏡技術對板栗「燕山早豐」的大小孢子發生及雌雄配子體發育過程進行觀察。結果：花藥壁的發育屬於基本型，其由表皮、藥室內壁、中層和絨氈層組成。絨氈層類型為腺質絨氈層。小孢子母細胞減數分裂過程中的胞質分裂方式為同時型，四分體的排列為四面體型，成熟花粉為2-細胞型，胚囊發育屬蓼型。每子房含有16~18個胚珠，胚珠倒生，雙珠被，厚珠心。受精之前，兩個極核融合形成一個次生核。成熟胚囊由7細胞組成。在子房中觀察到不正常的胚囊或敗育的胚珠。敗育的胚珠可能是導致「燕山早豐」結實率低的原因。

關鍵詞：板栗、大孢子發生、小孢子發生、雄配子體、雌配子體。

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INTRODUCTION

Chestnuts are one of the most important non-wood forest trees and have been cultivated for thousands of years (Payne et al. 1983). Chestnuts belong to the genus *Castanea* (of the Fagaceae), including 4 main economic species: *Castanea mollissima* Blume (Chinese), *C. dentate* (North American), *C. sativa* (European), and *C. crenata* (Japanese). Three species in this genus are native to China: *C. mollissima*, *C. sequinii* Dode, and *C. henryi* Rehd. & Wils (Huang 1998). *Castanea mollissima* thrives best at latitudes of 41°29′N~18°31′N. *Castanea mollissima* grows in tropical, temperate-continental, and temperate-maritime climates with mild winters and hot summers (Huang 1998). In China, this species is largely distributed in Jilin, Hebei, Shandong, Sichuan, Hubei, Anhui, Jiangsu, and Yunnan Provinces, and Hainan Island and is also found in Taiwan (Bounous and Marinoni 2005).

Chinese chestnut has long been recognized as a multipurpose species, because it represents an integral part of the economy in many areas, particularly in rural regions (Shi and Stösser 2005). Chestnut forests cover an area of over 20 x 10^6 ha. But low nut yields and an increase in alternate bearing are major problems for many chestnut farmers (Shi and Xia 2010). Although nut production of *C. mollissima* has received considerable attention in recent years, most studies of this species focused on the effects of ecological factors such as photosynthates (Proietti et al. 2000), water stress (Shi 2002), pollen donors (Hasegawa et al. 2009), and calcium nutrition (Wang 2011). In general, plant sexual reproductive processes could be related to fruit production (Guerra et al. 2011). However, only a few publications examined sexual reproduction in this genus. Sexual reproduction in *Castanea* was first described by Mc-

kay (1939, 1942), and additional studies of embryo development were reported (Zhang 1986, Xu et al. 1988, Shi and Stösser 2005, Zheng et al. 2009). Zhang (1986) examined the development of ovaries and embryos of the *C. mollissima* 'Taianhongli' cultivar. Xu et al. (1988) described development of the embryo sac and the process of fertilization and embryogenesis in *C. mollissima*. Shi and Stösser (2005) investigated the fertilization biology of *C. mollissima* 'Zaodali' and 'Jiujiazhong'. The processes of embryonic development and female gametophyte formation in *C. herryi* 'Huangzhen' were investigated by Zheng et al. (2009). The development of male and female gametophytes was considered to be key factors responsible for generating seeds. Therefore, those processes play prominent roles in maintaining the population size and regeneration of important species. Although these processes have been discussed for different *Castanea* species, the reasons for the low nut rate with sexual reproduction in the *C. mollissima* cultivar 'Yanshanzaofeng' are unknown, largely because many aspects of the reproductive behavior, especially male and female gametophyte development, remain elusive. The lack of basic knowledge of sexual reproduction is even more evident in chestnut trees. They are difficult, if not impossible, to grow in controlled conditions, and seasonal flowering is not easy to overcome. This creates strong experimental constraints and the necessity to carry out most experimental work in just a few months. It is generally accepted that better knowledge of the mechanisms of pollination and fertilization may result in better productivity (Pesson and Louveaux 1984). Therefore, knowledge of embryology's role in the development of reproductive organs in *C. mollissima* is essential to resolving these problems.

The Yanshanzaofeng cultivar has produced relatively high yields (459~612 kg/ha) of high-quality chestnuts in some commercial orchards, and possible causes of low fruit set or empty-bur formation were also investigated in some orchards. In the present study, the reproductive process was evaluated to ascertain the causes of low fruit set in open-pollinated flowers of this cultivar. The microsporogenesis, megasporogenesis, and development of male and female gametophytes in *C. mollissima* were observed using microscopic techniques, which provided embryological information on the reason for low fruit set and may be helpful in breeding research.

MATERIALS AND METHODS

Seedlings of *C. mollissima* 'Yanshanzaofeng' grown in Qianxi County (Hebei Province, China) (40°21′57″N, 118°12′17″E), at approximately 163 m in elevation were used in this study. This site is located in the warm temperate zone of a semi-humid region, with a mean annual precipitation of 744.7 mm and a mean annual temperature of 10.9℃.

Flowers of the chestnut cultivar Yanshanzaofeng from 2011 and 2012 were used in the study. All flower materials were obtained from a 12-yr-old tree selected for its good yield and because it was representative of the chestnut population in the Qianxi Chestnut Orchard. Samples were collected about every 3~5 d.

Light microscopy (LM)

Samples were fixed in formalin/glacial acetic acid/ 70% ethyl alcohol (FAA; 5: 5: 90, v/v) and stored at 4℃ prior to sectioning (Hsu et al. 2002). The flower materials were dehydrated in a graded ethanol series from water through 10-20-30-50-70-85-95-100% ethanol and embedded in paraffin wax (Chung and

Kuo 2005). Sections of 10 μm were prepared (using a Leica RM2265 microtome, Wetzlar, Germany) and then stained with hematoxylin-eosin Y or safranin O/fast green for photomicroscopic observation (Olympus BX-51 microscope, Tokyo, Japan). Some sections were stained with 0.5% decolorized aniline blue in 0.1 M K_3PO_4 for 3~12 h and observed under a fluorescence microscope (Olympus BX-51) to investigate callose deposition during microsporogenesis (Sogo and Tobe 2005).

Scanning electron microscopy (SEM)

Pollen grains were mounted on SEM stubs, coated with gold-palladium (Hitachi E-1010, Tokyo, Japan), examined with an SEM (Hitachi S-3400N), and photographed (Shi and Xia 2010).

RESULTS

Formation of the anther wall

Male flowers were tetrasporangiate anthers. In the early stage of development, archesporial cells (which were recognizable by their large volume with conspicuous nuclei) differentiated below the epidermis of the anthers (Fig. 1A). These cells divided periclinally to form outer primary parietal cells and inner primary sporogenous cells (Fig. 1A). The epidermal layer consisted of slightly elongated cells that gradually developed into compressed epidermal cells (Fig. 1B). Archesporical cells split by periclinal division to form an outer layer of primary parietal cells and inner primary sporogenous cells. Primary parietal cells repeatedly divided by periclinal and anticlinal division to form a subepidermal endothecium and middle layers (Fig. 1B). Cells of the outer layer again divided periclinally and anticlinally to form the massive nucellus. Inner cells formed sporogenous cells. Endothecial cells gradually elongated, and had acquired fibrous thickenings by the time of anthesis (Fig. 2G). The middle layers had a common histogenetic origin with the inner layer, and it persisted until the tetrad stage and then degenerated before forming 2-celled pollen grains (Fig. 2F). The innermost layer gave rise to the tapetum, which partly originated from the ground tissue near the connective tissue, and some of the tapetum cells had 1 or 2 prominent nuclei at the microspore mother cell meiosis stage. At the tetrad stage, tapetal cells elongated and were no longer in close contact but still remained in their original position (Fig. 2A). They began to degenerate at the stage of uninucleate microspores and had completely disintegrated by the binucleate pollen stage (Fig. 2F). The endothecium comprised 1 layer. The middle layers appeared to undergo further divisions to form 2 layers. The anther wall, prior to maturity, was usually comprised of 5 cell layers: a single epidermis, an endothecium, 2 middle layers, and the tapetum. Thus, the tapetum was of the glandular type, and wall formation conformed to the basic type as defined by Davis (1966).

Microsporogenesis and microgametogenesis

A row of sporogenous cells derived from archesporial cells, gave rise to a mass of microspore mother cells by several mitotic divisions (Fig. 1A). Simultaneously with changes taking place in the wall of the microsporangia, primary sporogenous cells underwent mitosis, forming secondary sporogenous cells, from which microsporocytes were derived. Microsporocytes were recognizable by their large volume, dense cytoplasm, and conspicuous nuclei (Fig. 1C). Microsporocytes underwent meiosis which involved 2 cell divisions. Meiosis I included the prophase (Fig. 1D), metaphase (Fig. 1E), anaphase, and telophase

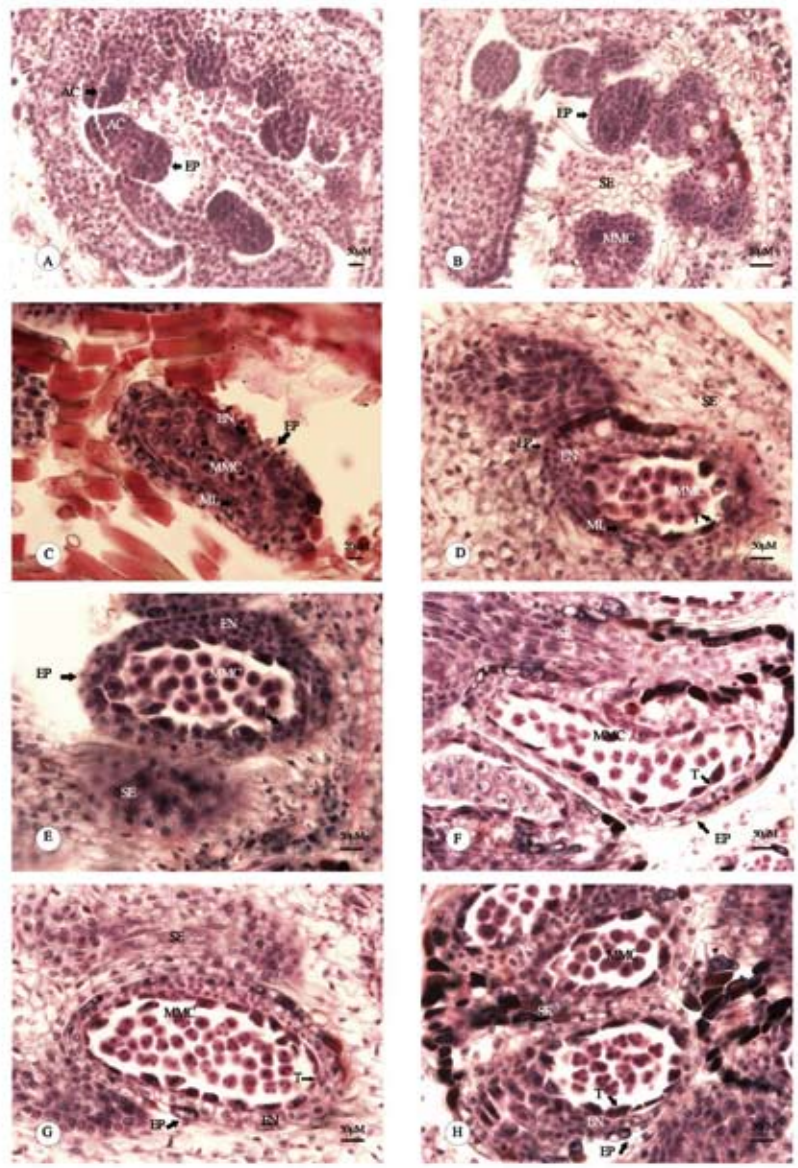

Fig. 1. Formation of microspores and development of male gametophytes in *Castanea mollissima*. (A) Archesporial cells which appeared at the corners of the young anther. (B) Anthers at the growing point of the catkin. (C) Details of an early microspore mother cell wall showing the microspore mother cell coat and the thin layer of the callose (epidermal layer, endothecium, 2 or 3 middle layers, and the inner tapetum). (D) Details of a late microspore mother cell wall at meiosis I prophase. (E) Details of a late microspore mother cell wall at meiosis I metaphase. (F) Details of a late microspore mother cell wall at meiosis I telophase. (G) Details of a late microspore mother cell wall at meiosis II metaphase. (H) Details of a late microspore mother cell wall at meiosis II telophase. AC, archesporial cell; EN, endothecium; EP, epidermis; ML, middle layers; MMC, microspore mother cell; MS, microspore; SE, septum; T, tapetum.

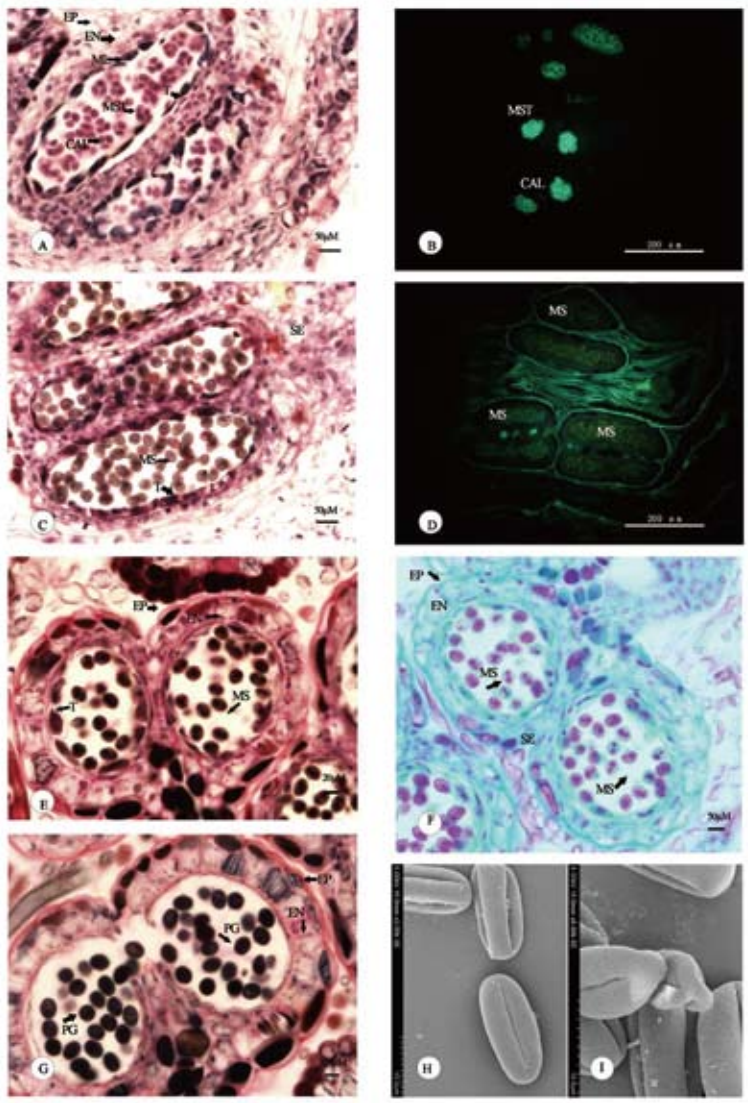

Fig. 2. Formation of microspores and development of male gametophytes in *Castanea mollissima*. (A) Details of microspores showing that microspore tetrads were tetrahedral and enclosed in a callose. (B) Details of the anther showing that microspore tetrads were enclosed in the callose layer, as seen under fluorescence microscopy. (C) The uninucleate stage of microspores of the anther. (D) Dissolution of the callose walls surrounding microsporogenous cells, as seen under fluorescence microscopy. (E) Anthers at the uninucleate microspore stage with a stretched epidermis, fibrous thickened endothecium, relic of middle layers, and a degenerated tapetum. (F) Anthers at the binucleate microspore stage. (G) Details of anthesis showing that the anther wall was composed of an epidermis, endothecium, and mature pollen grains. (H, I) Details of anthesis showing the mature pollen morphology under scanning electron microscopy. CAL, callose; EN, endothecium; EP, epidermis; ML, middle layers; MMC, microspore mother cell; MS, microspore; MST, microspore tetrad; PG, pollen grains; SE, septum; T, tapetum.

(Fig. 1F). As a result, a dyad of microspores was formed. A microspore tetrad was formed during prophase II, metaphase II (Fig. 1G), anaphase II, and telophase II (Fig. 1H). Most of the tetrads were tetrahedral (Fig. 2A). Callose deposition occurred at the onset of meiosis by pollen mother cells, reached a peak at metaphase II or anaphase II by enveloping the pollen mother cells or microtetrads (Fig. 2B), and had disappeared by the end of meiosis (Fig. 2D). Callose cycling (or cellulose synthesis) during microsporogenesis might play important roles in protecting the pollen mother cells from various environmental stresses and release of microspores from the microtetrads. Instead, simultaneous cytokinesis took place at the end of meiosis II of pollen mother cells.

Microspores were soon separated from each other and released from the tetrads. Microspores released from the tetrads had dense cytoplasm and conspicuous walls, with a prominent and centrally placed nucleus (Fig. 2C). As the central vacuole developed, the nucleus took a peripheral position. At this stage, uninucleate microspores underwent asymmetrical mitotic division (microspore mitosis) giving rise to 2 cells with distinct fates: vegetative and generative cells (Fig. 2E). Nuclei of vegetative cell were round and large and generally confined to the center of the pollen grain, whereas those of smaller generative cells were crescent-shaped and located close to the pollen wall (Fig. 2F). Mature pollen grains contained 2 cells with 3 germ pores (Fig. 2H, I). Anthers were dehiscent, and pollen grains were shed on approximately June 10.

Ovule development

Longitudinal sections of the ovary indicated that the flattened septa/placenta were compressed near the apex of the 9 locules (Fig. 3E). Ovules were attached in a shallow helical pattern within the ovary. Cells of the nucellar epidermal and subdermal layers divided anticlinally (Fig. 3A). The volume of nucellar cells beneath the top of the subdermal layers increased and differentiated into primary archesporial cells (Fig. 3B). Rapid mitosis in some nucellar epidermal cells around the base of the nucellus gave rise to the primordium of the inner integument (Fig. 3C). The outer integument primordium was initiated slightly later (Fig. 3C). Both the inner and outer integuments were composed of cells of 2 or 3 layers in thickness (Fig. 3C).

Both the inner and outer integuments quickly elongated and nearly enclosed the nucellus, except for its apex (Fig. 3C). In the nucellus, archesporial cells underwent periclinal division, forming primary parietal cells (Fig. 3C). Then, the volume of both integuments rapidly increased and completely enclosed the nucellus to form the micropyle. At this time, the ovules occupied a large part of the ovarian cavity, and the ovary was filled with darkly stained lignified trichomes (Fig. 3E).

There were approximately 16~18 ovules in the placenta of an ovary of *C. mollissima* (Fig. 3E). The ovaries were superior, bicarpellate, and unilocular or multilocular with parietal placentae. The integuments were initiated by periclinal and oblique divisions at the base of the nucellus. The integument reached the top of the nucellus and formed a micropyle by continuous division. Ovules in *C. mollissima* were anatropous. The structure of the ovule resembled that described for *Quercus* (Stairs 1964, Brown and Mogensen 1972, Borgardt and Pigg 1999, Borgardt and Nixon 2003, Deng et al. 2008). However, ovules that did not have viable embryo sacs either did not develop or were aborted. Due to insufficient pollination and fertilization, abortion of ovules occurred both before and

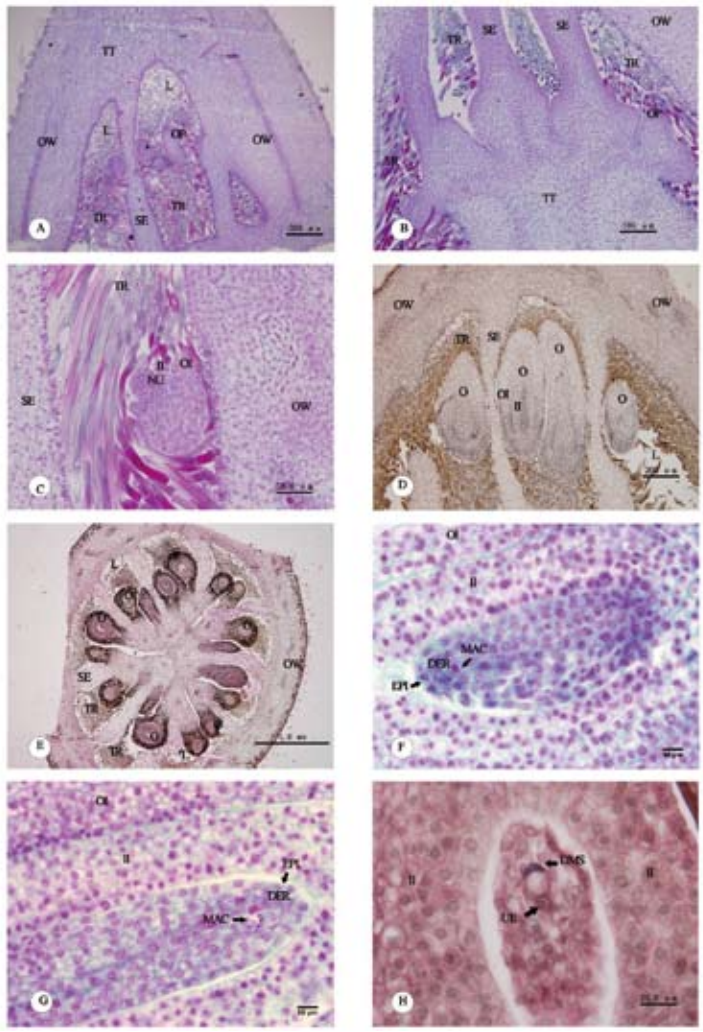

Fig. 3. Formation of megaspores and development of female gametophytes in *Castanea mollissima*. (A) Longitudinal section showing locules, transmitting tissue at the upper locule, septa, sessile placenta, ovule primordium, and unlignified trichomes of the locule. (B) Longitudinal section showing an enlarged locule filled with unlignified and lignified trichomes, elongated ovule primordial, septa, and placenta. (C) Longitudinal section showing initiation and development of the inner and outer integuments, and nucellus. (D) Longitudinal section showing ovules with the inner and outer integuments, a finger-like nucellus with an embryo sac, locule, and ovary wall. (E) Transverse section showing the ovary, 9 septa, and 9 locules. (F) Longitudinal section showing a megaspore mother cell at zygonema of the prophase I stage. (G) Longitudinal section showing a megaspore mother cell at diakinesis of the prophase I stage. (H) Longitudinal section showing a uninuclear embryo sac, degenerated megaspores at the chalazal end, and outer and inner integuments. DER, subdermal layer; EPI, epidermis; II, inner integument; L, locule; MAC, megaspore mother cell; NU, nucellus; OI, outer integument; OP, ovule primordial; OW, ovarian wall; SE, septum; TR, trichome; TT, transmitting tissue.

after development of the embryo sac (Fig. 4G, H). Normal and aborted ovules were hard to discriminate until the larger embryo sac appeared in normal ovules. Both integuments of aborted ovules were darkly stained with shrunken cells (Fig. 4G, H).

Megasporogenesis and megagametogenesis

A single archesporial cell differentiated under 1 layer of epidermal cells in the young nucellus (Fig. 3C). These archesporial cells directly functioned as a megasporocyte, which was easily distinguished from other cells by its large nucellus and dense cytoplasm (Fig. 3F). The archesporial cell did not form a parietal layer, but the nucellar epidermis divided periclinally, giving rise to a subdermal layer between the ovule epidermis and megaspore mother cell (Fig. 3G). Thus, the ovule was crassinucellate as defined by Davis (1966). The nucellus beneath the megaspore mother cells continuously elongated and developed into a mature finger-like shape (Fig. 3D).

The megasporocyte underwent meiosis to form the dyad of the megaspore. A megaspore tetrad was formed after meiosis, such a zygonema (Fig. 3F) and diakinesis (Fig. 3G). Three of the megaspores of the tetrad eventually degenerated, while chalazal 1 became functional (Fig. 3H). The functional megaspore successively developed into 2- (Figs. 4A-B), 4- (Fig. 4C), and 8-nucleate embryo sacs (Fig. 4D-F) after 3 meiotic divisions. Three cells (or nuclei) were grouped together at the micropylar end and constituted the egg apparatus (Fig. 4E). Cells were initially quite similar, then one of them became a fan-shaped egg cell and the other 2 developed into triangular-shaped synergid cells (Fig. 4D). Two central polar nuclei (positioned close to each other) were fused into a central cell before fertilization (Fig. 4D), while the antipodal cells were ephemeral and were at the chalazal end (Fig. 4F). Thus, the mode of embryo sac formation was of the *Polygonum* type (Davis 1966).

DISCUSSION

The anther of *C. mollissima* is microdiodanges. The mature anther wall layers are comprised of an epidermis, a 1-layered endothecium, 2 middle layers, and a single-layered tapetum. Cytokinesis during meiosis of its microspore mother cell is of the modified simultaneous type, and the tetrads are tetrahedral. The pollen grains are monocolpate and 2-celled when shed. Developmental characteristics of male gametophytes of *C. mollissima* are similar to those of *Cyclobalanopsis* (Lo and Huang 2005), *Castanea* (Mert and Soylu 2006), and *Quercus* species (Deng et al. 2008).

The tapetum during anther wall development is of the heteromorphic and glandular type. This type of tapetum was reported in some species of *Quercus* (Stairs 1964, Kaul 1985, Deng et al. 2008). At about the time the pollen tetrads form, the walls of tapetal cells become indistinct, and the tapetal cells degenerate at their original site. Tapetal cells are completely degenerated by the 1-nucleate pollen stage. In addition, callose deposition occurs at the onset of meiosis of pollen mother cells but disappears by the end of meiosis. Callose cycling (or cellulose synthesis) during microsporogenesis might play important roles in protecting pollen mother cells from various environmental stresses and in releasing microspores from the microtetrads (Li and Ma 2006).

Cytoplasmic male sterility might be an important factor influencing chestnut production (Mckay 1939, Jaynes 1963, Omura and Akihama 1980, Soylu 1992, Sisco et al. 2001,

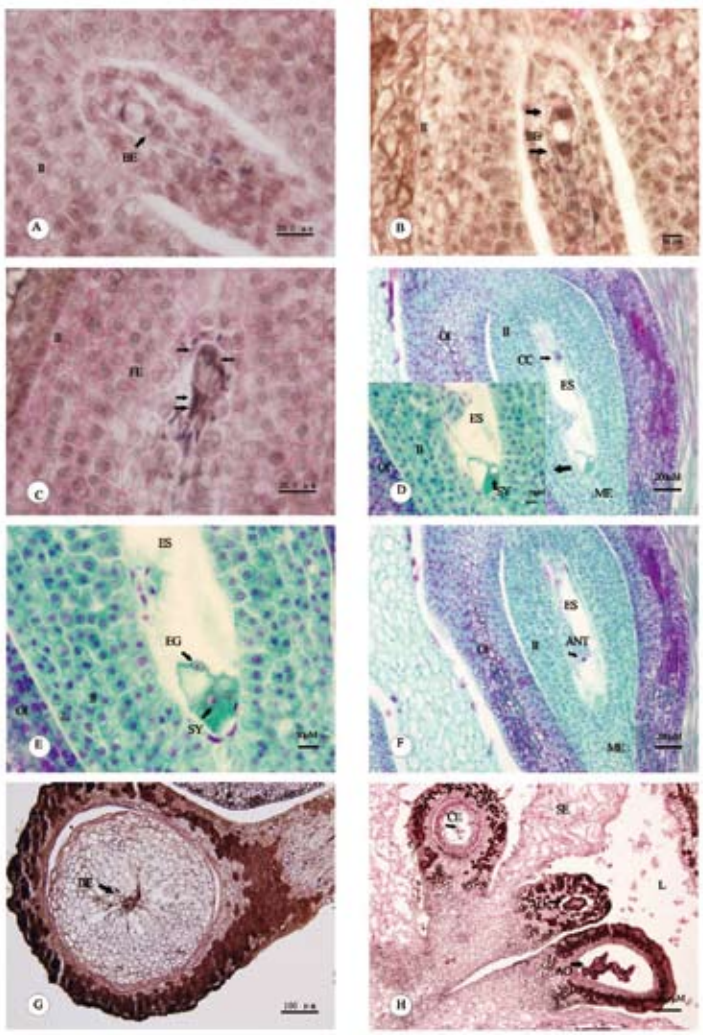

Fig. 4. Formation of megaspores and development of female gametophytes in *Castanea mollissima*. (A, B) Longitudinal section showing a binuclear embryo sac, and outer and inner integuments. (C) Longitudinal section at the 4-nucleate embryo sac stage. (D-F) Developmental stages of a mature embryo sac. (D) Longitudinal section showing another synergid cell at the micropylar end, 2 central polar nuclei fused into a central cell before fertilization, and the inner and outer integuments. (E) Longitudinal section showing an egg cell at the micropylar end, a synergid cell, and the inner and outer integuments. (F) Enlarged embryo sac showing antipodal cells at the chalazal end, postament, and the inner and outer integuments. (G, H) Longitudinal section showing an abortive ovule, degenerated embryo sac, cavity of the embryo sac, locule, and septum. ANT, antipodal cells; AO, abortive ovule; BE, binuclear embryo sac; CC, central cell; CE, cavity embryo sac; DE, degenerate embryo sac; DMS, degenerate megaspores; EG, egg cell; ES, embryo sac; FE, 4-nucleate embryo sac; II, inner integument; L, locule; ME, micropylar end; MT, megaspore tetrad; O, ovule; OI, outer integument; OW, ovarian wall; PO, postament; SY, synergid; UE, uninuclear embryo sac.

Mert and Soylu 2006). Cytoplasmic male sterility is usually accompanied by abnormal callose deposition or anomalies of the tapetum (Kaul 1988). In our anatomical investigation, we did not find any abnormal callose deposition or anomalies in the tapetum during microsporogenesis in *C. mollissima* Yanshanzaofeng, indicating that male gametes were fertile, male sterility was not the major cause of the low seed set in the Yanshanzaofeng cultivar, and thus 'Yanshanzaofeng' was considered to be pollenizers.

This study of the Yanshanzaofeng cultivar showed that development of the embryo sac was of the *Polygonum* type as previously described (Botta et al. 1995, Shi and Stösser 2005). Ovules were anatropous, bitegmic, and crassinucellate, and had an obturator. Development of the internal and outer integuments was similar to that of other species of *Castanea* (Zhang 1986, Xu et al. 1988, Botta et al. 1995, Shi and Stösser 2005, Zheng et al. 2009).

It is generally known that normal ovule development is important for high fruit set in orchards (Jia et al. 2008). It was reported that in *C. mollissima*, each ovule might be fertilized and be capable of producing a seed (McKay 1942). It was proposed that all of the ovules that develop a normal embryo sac are potential seeds (Mogensen 1975). In our anatomical experiment, although most of the female gametophytes developed normally, some ovules were abortive in the Yanshanzaofeng cultivar. The absence of embryo sacs and the occurrence of empty embryo sacs accounted for abortion in other ovules. The observed anomalies in *C. mollissima* were also consistent with previous observations (Jialin et al. 1990, Botta 1995, Shi and Stösser 2005). Within the Fagaceae, characteristics of non-developed ovules of *C. mollissima* were similar to those of *Quercus* species (Liao 1970, Mogensen 1975, Kaul 1985, Borgardt and Pigg 1999, Borgardt and Nixon 2003, Deng et al. 2008). To our knowledge, the ovule is the source of the megagametophyte and the progenitor of the seed (Reiser and Fischer 1993). Poor fruit set has been attributed to undesirable environmental conditions or male or female sterility (Julian et al. 2010, Guerra et al. 2011). However, in our study, we found that Yanshanzaofeng grew under good hydrothermal conditions in orchards, and also exhibited male fertility. Ovules in each ovary always formed a 1-seeded fruit or nut within a single shell, and bearing single-seeded nuts seems to be related in part to the high occurrence of anomalies of the embryo sac (Mckay 1942, Botta 1995). Abortive ovules resulted in the formation of empty seeds or cupules (Zheng CL et al. 2009). The main cause of the low yield is a high proportion of empty cupules or fewer than 3 nuts per cupule (Shi and Xia 2010). Thus, the low fruit set in Yanshanzaofeng could be partially explained by the abortive ovules during female gametophyte development (Shi and Stösser 2005), and a high percentage of abortive ovules was identified as the major factor causing female sterility and possibly influenced nut production in Yanshanzaofeng orchards. But future studies should be performed to delineate whether the abortive ovules were caused by resource limitations or programmed cell death.

CONCLUSIONS

This study provides basic information on different sexual reproductive aspects of *C. mollissima* and could shed light on the embryogenesis in this species. In *C. mollissima*, microgametogenesis results in binucleate pollen as was observed in other genera in the Fagaceae, and macrogametogenesis belongs to the *Polygonum* type. Abnormal embryo

sacs or abortive ovules were observed in the ovaries, suggesting that the same cellular mechanisms might act in somatic embryogenesis as well as in embryo rescue and induction of embryogenesis (Viejo et al. 2010). A large number of abortive ovules may influence yields of *C. mollissima* 'Yanshanzaofeng', and further studies are needed to corroborate these results.

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LITERATURE CITED

Borgardt SJ, Nixon KC. 2003. A comparative flower and fruit anatomical study of *Quercus acutissima*, a biennial-fruiting oak from the *Cerris* group (Fagaceae). Am J Bot 90(11):1567-84.

Borgardt SJ, Pigg KB. 1999. Anatomical and developmental study of petrified *Quercus* (Fagaceae) fruits from the Middle Miocene, Yakima Canyon, Washington, USA. Am J Bot 86(3):307-25.

Botta R, Vergano G, Me G, Vallania R. 1995. Floral biology and embryo development in chestnut (*Castanea sativa* Mill.). HortScience 30(6):1283-6.

Bounous G, Marinoni DT. 2005. Chestnut: botany, horticulture, and utilization. Hort Rev (31):291-347.

Brown RC, Mogensen HL. 1972. Late ovule and early embryo development in *Quercus gambelii*. Am J Bot (59):311-6.

Chung JD, Kuo SR. 2005. Reproductive cycles of *Calocedrus formosana*. Taiwan J For Sci 20(4):315-29.

Davis GL. 1966. Systematic embryology of the angiosperm. New York: Wiley. p 283-505.

Deng M, Zhou ZK, Chen YQ, Sun WB. 2008. Systematic significance of the development and anatomy of flowers and fruit of *Quercus schottkyana* (subgenus *Cyclobalanopsis*: Fagaceae). Int J Plant Sci 169(9):1261-77.

Guerra ME, Wűnsch A, López-Corrales M, Rodrigo J. 2011. Lack of fruit set caused by ovule degeneration in Japanese plum. J Am Soc Hort Sci 136(6):375-81.

Hasegawa Y, Suyama Y, Seiwa K. 2009. Pollen donor composition during the early phase of reproduction revealed by DNA genotyping of pollen grains and seeds of *Castanea crenata*. New Phytol 182:994-1002.

Huang HW. 1998. Review of current research of the world *Castanea* species and importance of germplasm conservation of China native *Castanea* species. J Wuhan Bot Res 16(2):171-6. [in Chinese].

Hsu HW, Kuo SR, Chuang NJ, Liang YC. 2002. Phenology of growth and development of strobili of *Taiwania cryptomerioides* Hay. Taiwan J For Sci 17(2):241-55.

Jaynes RA. 1963. Male sterility in *Castanea*. Genetics today: Proceeding of the XI International Congress of Genetics. The Hague, Netherlands: Oxford Press, p 203. [Abstract].

Jia HJ, He FJ, Xiong CZ, Zhu FR, Okamoto G. 2008. Influences of cross pollination on pollen tube growth and fruit set in Zuili plums (*Prunus salicina*). J Integr Plant Biol 50(2):203-9.

Yao JL, Xia RX, Ma MT, Fu WW. 1990.

Study on factors affecting empty bur formation of chestnut (*Castanea mollissima* Blume.). II. Embryology of empty bur. J Huazhong Agric Univ. 8(3):248-53. [in Chinese with English abstract].

Carme J, Herrero M, Rodrigo J. 2010. Flower bud differentiation and development in fruiting and non-fruiting shoots in relation to fruit set in apricot (*Prunus armeniaca* L.). Tree 24:833-41.

Kaul MLH. 1988. Male sterility in higher plants. Berlin: Springer-Verlag. p 3-97.

Kaul RB. 1985. Reproductive morphology of *Quercus* (Fagaceae). Am J Bot 72(12):1962-77.

Li WD, Ma FS. 2006. Reproductive biology of sexual hybridization in woody plants: an atlas. Beijing, China: Science Press. p 1-16.

Liao JC. 1970. Morphological studies on the flowers and fruits of the genus *Quercus* in Taiwan. Mem Coll Agric Natl Taiwan Univ. 11: 22-74.

Lo HC, Huang TM. 2005. Microsporogenesis in *Cyclobalanopsis glauca*. J Exp For Natl Taiwan Univ. 19(1):55-68. [in Chinese].

Mckay JW. 1939. Male sterility in *Castanea.* Am Soc Hort Sci Proc (37):509-10.

Mckay JW. 1942. Self-sterility in the Chinese chestnut (*Castanea mollissima*). Proc Am Soc Hort Sci (41):156-61.

Mert C, Soylu A. 2006. Flower and stamen structures of male-fertile and male-sterile chestnut (*Castanea sativa* Mill.). J Am Soc Hort Sci 131(6):752-9.

Mogensen HL. 1975. Ovule abortion in *Quercus* (Fagaceae). Am J Bot 62:160-2.

Omura M, Akihama T. 1980. Male sterility in chestnuts: a tentative plan for the seed propagation of fruit trees. Gamma Field Symp (19):77-89.

Payne JA, Jaynes RA, Kays SJ. 1983. Chinese chestnut production in the United States: practice, problems, and possible solutions. Econ Bot 37(2):187-200.

Pesson P, Louveaux J. 1984. Pollinisation et productions végétales. Paris: Inra. p 663.

Proietti P, Palliotti A, Famiani F, Antognozzi E, Francesco F, Andreutti R, Frenguelli G. 2000. Influence of leaf position, fruit and light availability on photosynthesis of two chestnut genotypes. Sci Hort-Amsterdam (85):63-73.

Reiser L, Fischer LR. 1993. The ovule and the embryo sac. Plant Cell 5:1291-1301.

Shi Z, Stösser R. 2005. Reproductive biology of Chinese chestnut (*Castanea mollissima* Blume.). Eur J Hort Sci 70(2):96-113.

Shi ZG, Xia L. 2010. Stigmatic morphology of Chinese chestnut (*Castanea mollissima* Blume.). HortScience 45(6):981-3.

Shi ZJ. 2002. Study of adaptation metabolism of chestnut. etc species on water stress and pod kinetic characteristic of chestnut leaves. Fujian, China: Fujian Agriculture and Forestry Univ. p 1-48. [in Chinese with English abstract].

Sisco PH, Hebard FV, Shi Y, Kubisiak TL. 2001. Cytoplasmic male sterility resulting from interspecific crosses in chestnut (*Castanea* spp.). Plant and Animal Genome IX Conference. San Diego, CA: Forest Tree Genome and IUFRO Working Party on Genomics.

Sogo A, Tobe H. 2005. Intermittent pollen-tube growth in pistils of alders (*Alnus*). PANS 102(24):8770-5.

Soylu A. 1992. Heredity of male sterility in some chestnut cultivars (*Castanea sativa* Mill.). Acta Hort 317:181-5.

Stairs GR. 1964. Microsporogensis and embryogenesis in *Quercus*. Bot Gaz 125(2):115-21.

Viejo M, Rodríguez R, Valledor L, Pérez M, Cañal MJ, Hasbún R. 2010. DNA methylation during sexual embryogenesis and implications on the induction of somatic embryogenesis in *Castanea sativa* Miller. Sex Plant Reprod (23):315-23.

Wang J. 2011. Study on calcium nutrition of *Castanea mollissima* Blume. Beijing, China:

Beijing Forestry Univ. p 1-47. [in Chinese with English abstract].

Xu HL, Cao HJ, Li TQ. 1988. Study on the embryology of chestnut (*Castanea mollissima* Blume.) I. The development of ovules and the embryo sac, fertilization and embryogenesis. J Beijing For Univ. 10(1):10-20. [in Chinese with English abstract].

Zhang HY. 1986. On the development of ovary and embryo of chestnut (*Castanea mollissima* Blume.). J Shangdong Agric Univ. 17(2):19-25. [in Chinese with English abstract].

Zheng CL, Pan DM, Zhang DC, Wu XZ, Lv JM, Yu XQ, et al. 2009. Studies on regularities of growth and development of chinquapin (*Castanea henryi*) fruits. ISHS Acta Horticulturae 844: IV International Chestnut Symposium. Beijing, China: International Society for Horticultural Science.

Zheng CL, Yu XQ, Pan DM. 2009. Process of embryonic development and female gametophyte in chinquapin (*Castanea henryi* Rehd & Wils). J Fujian Agric For Univ. 38(6):585-9. [in Chinese with English abstract].