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Megasporogenesis and Development of Female Gametophyte in Chinese Chestnut (*Castanea mollissima*) Cultivar 'Yanshanzaofeng'

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Abstract

Chinese Chestnut (*Castanea mollissima* Blume) is used as a non-wood forest tree and well-known for its edible value. Some chestnut cultivars are particularly prone to erratic fruit set showing very low or even empty cupule for unknown reasons. To investigate the causes of lack of fruit set in them, the megasporgensis and development of female gametophytes of *C. mollissima* 'Yanshanzaofeng' were evaluated by microscopy. The results are showed that 'Yanshanzaofeng' was diclinous with polycarpous compound pistils, axile placentas, and commonly two anatropous ovules in each loculament. An archesporial cell in the nucellus tissue would directly develop into a megasporocyte that subsequently formed the megaspore tetrads after meiosis. The functional megaspore near the chalazal end developed into mature embryo sac, which was of *Polygonum* type. After double fertilization, the proembryo developed into a mature embryo going through the clavate, globular, heart, torpedo and cotyledon stages. The embryo development conformed to the *Onagrad* type and the endosperm development was nuclear. Abnormal embryo sacs or abortive ovules were observed in the ovary. Our results suggested that the abortive ovules is a likely cause of low seed set in *C. mollissima* 'Yanshanzaofeng'.

Keywords: Megasporogenesis; Female gametophyte; Abortive ovule; Chestnut

Introduction

Chestnut has been cultivated as an important fruit crop since ancient times (Payne *et al*., 1983). It is widely distributed in the temperate zones of the Northern hemisphere (Ertan, 2007), including East Asia, North American and Europe, particularly in China (Serdar *et al*., 2011). Chinese chestnut (*Castanea mollissma* Blume) is an economically important cultivated species being low in fat and good eating quality with generally medium in size (Wang *et al*., 2012). Chinese chestnut has been introduced into many countries due to its best adaptability for good cold hardiness and adequate tolerance to chestnut blight (Bounous and Marinoni, 2005). In addition, Chinese chestnut plays an important role in keeping soil and greening barren hills (Martín *et al*., 2012).

Understanding the reproductive biology of the species in order to make accurate predictions about patterns of chestnut production, will be useful in both seed orchard management and increasing nut production in natural or planted stands (Feijó *et al*., 1999). The research on reproductive biology of the plant is considered as the key factors responsible for generating seeds (Guerra *et al*., 2011). Thus, the developmental process of megasporogenesis and female gametophytes plays a prominent role in contributing to population maintenance and regeneration of important species (Qu *et al.*, 2010). In recent years, considerable attention has been paid to the embryology of *Castanea* species (McKay, 1940, 1942; Zhang, 1986; Xu, 1988; Botta *et al*., 1995; Shi and Stösser, 2005; Zheng *et al*., 2009). Although these sexual processes have been discussed in different *Castanea* species, the reasons for low fruit rate of sexual reproduction in *C. mollissima* remain unknown or incomplete. Moreover, there was no concomitant description on anatomic characterization of cultivar 'Yanshanzaofeng'. In general, many of the cultivated varieties are sterile or produce only a very few viable seeds. One possible cause being the morphological abnormalities observed in flowers. Therefore, detailed studies of this cultivar's embryogenesis development is essential for solving the problem.

In this study, the process of chestnut sexual production is described to provide a complete description of megasporgensis and development of female gametophytes. Our aim was not only to identify the main causes of low fruit set in open-pollinated flowers of this cultivar, but also to provide basic information that will be helpful in the development of chestnut production protocols to enhance nut quality and yield.

Materials and Methods

Pistillate flowers of the chestnut cultivars *C. mollissma* 'Yanshanzaofeng' at successive developmental stages were collected from a 12-year-old tree in Qianxi Chestnut

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Orchard (Hebei Province, China) (40°21′57″N, 118°12′17″E), at approximately 163m above sea level. *C. mollissma* 'Yanshanzaofeng' was first selected in Qianxi county and became an improved varieties in 1989. It was famous for ripening in the early September and developed so quickly that it covered about 45,000 hectare cultivated area in Qianxi county (Zou *et al*., 2013). The materials were collected every 3-5 days in June-August and being representative of the chestnut population. The pistillate flowers were fixed in FAA (70% alcohol: formalin: acetic acid = 90: 5: 5, v/v) for 24 h at room temperature (Chehregani and Sedaghat, 2009), dehydrated through an ethyl-alcohol series (Chehregani *et al*., 2011), embedded in paraffin with a 58-60^{o}C melting point, and sectioned at a thickness of 10 µm by a microtome (Leika RM2265, Germany). The sections were stained with Heidenhain's iron aium haematoxylin (Yuan *et al*., 2011; Guo *et al*., 2014). Observations and photographs of sections were carried out using an BX-51 and BX-61 microscope (Olympus, Japan).

Results

Development of Ovule

Pistil flower emergence and development lasted for 4 months — from May to August. The ovule primordiaum began development in the early June (Table 1). Approximately in mid of June, the cells of the nucellar epidermal layer and the subdermal layer divided anticlinally. Some nucellar epidermal cells around the base of the nucellus resulted in the primordium of the inner integument by rapid mitosis (Fig. 1A). The inner integument was initiated from dermal cells at the base of the ovule primordium earlier than the outer one. The outer integument always grew more slowly than the inner. The integument enclosed the nucellus and formed the micropyle (Fig. 1A). The nucellus beneath the megaspore mother cells continuously elongated and developed into mature finger-like shape (Fig. 1B). In the nucellus, the archesporial cell, without periclinal division, formed a primary parietal cell that directly developed into a megaspore mother cell on the later-June (Fig. 1C). Thus, the ovules in *C. mollissma* were anatropous, bitegmic and crassinucellate. In most other Fagaceae, the structure of the ovules of *C. mollissma* was similar to species of *Quercus*.

Development of Megasporogensis and Megagametogenesis

On June 20, a typical megaspore mother cell was first observed which could be easily recognized by its large nucleus with dense cytoplasm (Fig. 1C) (Table 1). After meiosis, I and II, a megaspore tetrad was formed. Usually, near the chalazal megaspore developed into the functional megaspore while the other three megaspores were eventually degenerated (Fig. 1D). The first division of the functional megaspore, the nucleus resulted in the formation of the two-nucleate stage (Fig. 1E). After these, two nuclei underwent a second meiotic division, giving rise to form a four-nucleate female gametophyte (Fig. 1F). An additional mitosis of these nuclei, an eight-nucleate megagametophyte was formed in the early-July (Fig. 1G-I). An egg cell and synergid cell that consisted of the egg apparatus were located at the micropylar pole, but the synergid cell were degenerated very soon (Fig. 1H). Two polar nuclei met in the middle of the embryo sac and fused to form a single, large, central cell before fertilization (Fig. 1G). Three antipodal cells were located at the chalazal pole and generally degenerated soon (Fig. 1I). Thus, this development of embryo was sac 8-nucleate *Polugonum* type.

Table 1: Phenology of embryogensis development in *C. mollissima*

Developmental stage	Approximate time when stage began	Time before (-) or after (+) pollination
Ovule primordiaum	Early June	-2 week
Inner and outer integument	Middle June	-1 week
Pollination	Middle June	
Megaspore mother cell	Later June	+1 week
One-nucleate embryo sac	Later June	+1 week
Eight-nucleate embryo sac	Early July	+2 week
Fertilization	Early July	+2 week
Free nuclear endosperm	Early July	+3 week
Zygote	Middle July	+3 week
Globose embryo	Middle July	+4 week
Torpedo-shaped embryo	Later July	+5 week
Cotyledon-shaped embryo	Later July	+6 week
Mature embryo	Early August	+7 week

Development of Endosperm

In the July 6 samples, after double fertilization, the zygote remained undivided which was located at the micropylar pole (Table 1). Although we did not see fertilization of the egg cell, presuming fertilization occurred ~3 d earlier, based on the formation of large primary endosperm nucleus (Fig. 2A). In the sample collected on July 9, repeated divisions of the primary nuclei developed into the free nuclear endosperm (Table 1). The free nuclei were peripheral to the central area of the embryo sac by dense cytoplasm connections (Fig. 2C). Therefore, the endosperm conformed to the nuclear type.

The zygote was at dormancy stage during and after the development of the free nucleate endosperm, whereas the inner integuments progressively degenerated (Fig. 2C). The endosperm eventually became cellular on the July 18 (Fig. 2D).

Development of Embryo

In the early-July, the division of zygote was delayed until the free nuclear endosperm was formed (Fig. 2A-C).

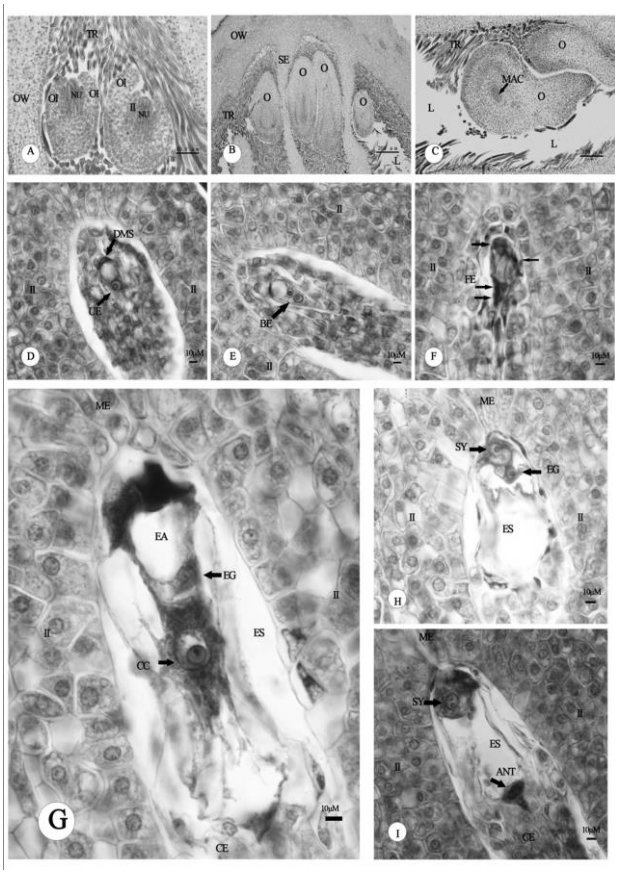

Fig. 1: Development of *C. mollissima* female gametophyte. A, Initiation, development of inner and outer integument, and nucellus. B, Figner-like nucellus with embryo sac. C, Megaspore mother cell. D, Uninuclear embryo sac. E, Binuclear embryo sac. F, Four-nucleate embryo sac. G, Egg cell at the micropylar end, two central polar nuclei fused into a central cell before fertilization. H, An egg cell at the micropylar end, and a synergid cell. I, A synergid cell at the micropylar end and antipodal cells at the chalazal end. (ANT = antipodal cells; BE = binuclear embryo sac; CC = central cell; DMS = degenerate megaspores; EG = egg cell; ES = embryo sac; FE = four-nucleate embryo sac; II = inner integument; L = locule; MAC = megaspore mother cell; NU = nucellus; OI = outer integument; OW = ovarian wall; SE = septum; TR = trichome; ME = micropylar end; O = ovule; OI = outer integument; OW = ovarian wall; SY = synergid; TR = trichome; UE = uninuclear embryo sac.) Scale bars: A = 50 µm; B = 200 µm; C = 100 µm; D-I= 10 µm

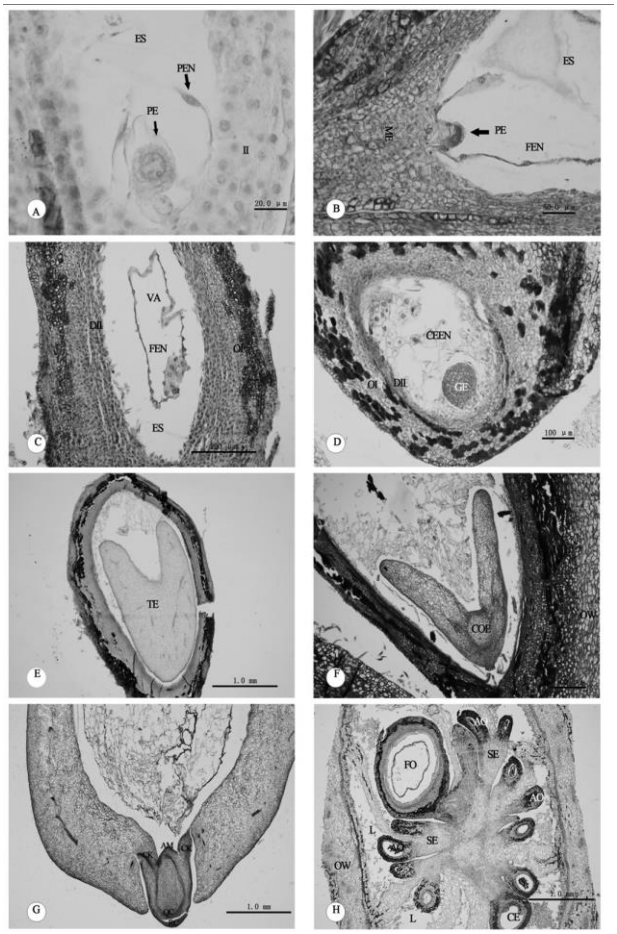

Fig. 2: Development of *C. mollissima* embryo. A, A two-cell proembryo and primary endosperm nucleus. B, The clavate proembryo and primary endosperm nucleu. C, The free endosperm, and the degenerated inner integument. D, The globular embryo, and the cellular endosperm. E, The torpedo embryo. F, The cotyledon embryo. G, The mature embryo, the apical meristem, the cotyledonary knots, the quiescient center and the root cap. H, The abortive ovule, functional ovule, degenerated embryo sac, and cavity of embryo sac. (AO = abortive ovule; AM = apical meristem; CEEN = cellular endosperm; CK = cotyledonary knots; COE = cotyledon embryo; DII = degenerated inner integument; ES = embryo sac; FO = functional ovule; GE = globular embryo; L = locule; ME = micropylar end; OI = outer integument; OW = ovary wall; PEN = primary endosperm nucleus; PE = proembryo; QC = quiescient center; RC = root cap; TE = torpedo embryo; VA = vacuole). Scale bars: A =20 µm; B =50 µm; C =200 µm; D and F=100 µm; E, G and H =1.0 mm

After a well-developed endosperm, the zygote resumed rapid growth by meiotic division later in July. Repeated divisions of all cells resulted in a clavate pro embryo (Fig. 2B). During further development, the embryo became young globose embryo (Fig. 2D), torpedo-shaped (Fig. 2E) and finally cotyledon-shaped (Fig. 2F). The mature embryo was formed around the early August (Table 1). It consisted of radicle, hypocotyl, piumule, and cotyledon, surrounded by a mass of partially digested endosperm (Fig. 2G). Therefore, this embryogeny conformed to the *Onagrad* type.

Abortion of the Other Ovules during Development

A total of 16~18 ovules were found from the ovary axis, but in the same ovary, ovules were at several stages of development. Generally, at least one embryo sac successfully developed in the ovary, but only one always matured into a seed. In the developmental process of the ovules that did not have visible embryo sacs were either undeveloped or aborted. Due to insufficient pollination and fertilization, abortion of ovules happened both before and after the development of the female gametophyte. It was hard to discriminate between the normal and aborted ovules unless the larger embryo sac appeared in the ovules (Fig. 2H). When the endosperm nucleus was present in the center of the enlarging embryo sac around 18 July (Fig. 2H), suggesting that the other ovules showed signs of abortion. The aborted ovules were due to degenerated embryo sac with darkly stained or empty embryo sac (Fig. 2H). Thus, it was reasonable to assume that these ovules were going to abort.

Discussion

The method of embryogenesis in *C. mollissima* was the same as those reported for other species of in terms of the anatropous, bitegmic and crassinucellate ovules, and the *Polygonum* type embryo sac (Xu *et al*., 1988; Botta *et al*., 1995; Zheng *et al*., 2009). Embryo development in Fagaceae follows the *Onagrad* type, as previously described in *Castanen* (Botta *et al*., 1995; Shi and StÖsser, 2005).

It is generally known that the normal ovule development is important for high fruit set in orchards (Jia *et al*., 2008). It was reported that in *C. mollissma* 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. The absence of embryo sacs and the occurrence of empty embryo sacs accounted for abortion in other ovules. The observed anomalies in *C. mollissima* also were consistent with previous observations (Yao *et al*., 1990; Shi and StÖsser, 2005). 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 an undesirable environmental conditions or male sterility or female sterility (Julian *et al*., 2010; Huang *et al*., 2011; Guerra *et al*., 2011). However, in our study, we found 'Yanshanzaofeng' not only grew under a good hydrothermal condition in orchards, but also exhibited male fertility (Zou *et al*., 2013). The low fruit set in 'Yanshanzaofeng' could be partially explained by abortive ovules in the female gametophytes development (Shi and Stösser, 2005), and a high percentage of abortive ovules was identified as the major factor causing female sterility and possibly influenced the nut production in 'Yanshanzaofeng' orchards. But further studies should be performed to delineate whether the abortive ovules were caused by failure fertilization or programmed cell death in the future.

In conclusion, this study provided basic information on the sexual reproduction aspects of *C. mollissma* 'Yanshanzaofeng'. In *C. mollissma*, the macrogametogenesis belongs to the *Polygonum* type, the endosperm conforms to the nuclear type and embryo development follows the *Onagrad* type as has been observed in other genera in *Castanea*. Abnormal embryo sacs or abortive ovules were observed in the ovary, suggesting that the same cellular mechanisms might act in somatic as well as in embryo rescue and embryogenesis induction. A large number of abortive ovules may influence yield in *C. mollissma*, and further studies are needed to corroborate these results.

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