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A pattern of unique embryogenesis occurring *via* apomixis in *Carya cathayensis*

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Abstract

Apomixis represents an alteration of classical sexual plant reproduction to produce seeds that have essentially clonal embryos. In this report, hickory (*Carya cathayensis* Sarg.), which is an important oil tree, is identified as a new apomictic species. The ovary has a chamber containing one ovule that is unitegmic and orthotropous. Embryological investigations indicated that the developmental pattern of embryo sac formation is typical polygonum-type. Zygote embryos were not found during numerous histological investigations, and the embryo originated from nucellar cells. Nucellar embryo initials were found both at the micropylar and chalazal ends of the embryo sac, but the mature embryo developed only at the nucellar beak region. The mass of the nucellar embryo initial at the nucellar beak region developed into a nucellar embryo or split into two nucellar proembryos. The later development of the nucellar embryo was similar to the zygotic embryo and progressed from globular embryo to heart-shape embryo and to cotyledon embryo.

Additional key words: adventitious embryony, female gametophyte, hickory, nucellar embryo.

Introduction

The term apomixis was first introduced by Winkler (1908) to refer to the "substitution of sexual reproduction by an asexual multiplication process without nucleus and cell fusion". Apomixis in flowering plants is the asexual formation of seed directly from the maternal tissues of the ovule, avoiding meiosis and fertilization (Bicknell and Koltunow 2004). Seeds resulting from apomixis are genetically identical to maternal plants, which is important for promoting the breeding process through fixing hybrid vigor and arousing researchers' interest (Hanna 1995, Koltunow *et al*. 1995a, Savidan 2000). Apomixis has been described in more than 400 flowering plant species belonging to over 40 families (Carman 1997). Among fruit tree species, only a few show this phenomenon and these fruits generally propagate vegetatively, including citrus and mango (Mo *et al*. 2005). Apomixis can be divided broadly into three types: *1*) adventitious embryony, *2*) a sporophytic type, or *3*) two gametophytic types diplospory and apospory (Peggy 2006, Koltunow 1993). Adventitious embryos can arise from two different tissues of the mature ovule, namely the nucellus and the inner integument (Lakshmanan and Ambegaokar 1984, Miles 2007). The form of the nucellus is common and this has been described in detail in *Citrus*, which is a model system for studying nucellar embryony.

Hickory (*Carya cathayensis* Sarg.), a member of the family *Juglandaceae*, is an important oil tree of high economic value (Li 2003). In walnut, apomixis was confirmed (Loiko 1990, Asadian and Pieber 2005), and later it was investigated in different cultivars using isoenzyme analysis (San and Dumanoğlu 2006, Wu *et al*. 2006). The mechanisms of apomixis in walnut have been reported as adventitious embryony (Valdiviesso 1990), apospory (Terziiski and Stefanova 1990) or diplospory (Sartorius and Stosser 1991). Sartorius and Stösser (1997)

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Abbreviations: AFLP - amplified fragment length polymorphism; DAP - days after pollination; FCSS - flow cytometric seed screening; SSR - simple sequence repeat.
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and Chen *et al*. (2000) reported development of apomictic embryos from the egg cell in different walnut cultivars. Schanderl (1964) reported that the development of apomictic embryos in walnut was diplosporic and not by nucellar embryony. During our previous study with hickory (Wang *et al*. 2010), we observed a certain proportion of polyembryonic seedlings (9.04 %). Furthermore, we conducted a hybrid experiment between hickory and pecan (*C. illinoensis*). The hybrids showed variations in fruit shape, pericarp colour and seed size and mass. Using short-sequence repeat (SSR) and amplified fragment length polymorphism (AFLP), differences between the hybrids and parents were analyzed. Differential bands were found between the paternal and maternal parents, as well as between the paternal parent and the progeny, but there was no difference between the maternal parent and the progeny or amongst the progeny (Wang *et al*. 2010). In addition, flower bud differentiation in hickory was studied in detail (Huang *et al*. 2006, 2007). The aim of the present study was to investigate the female gametophyte and embryo development in hickory to understand the mechanisms of its reproductive processes.

Materials and methods

A 50-year-old adult hickory (*Carya cathayensis* Sarg.) tree from Lin'an city, Zhejiang province (China) was chosen as the mother tree. The female flowers were bagged when the stigma began to turn red on 11 May 2010. During the pollination period, the female flowers were pollinated with mixed pollen from the mother tree and surrounding trees on 17 May. One week later the paper bags were removed once male inflorescences had faded.

When florets were visible (approximately 10 mm), male flowers were collected every 4 d, and female flowers were collected at different developmental stages according to the colour of the stigma. After pollination, female flowers and young fruits were collected every day during the first 7 d, and then at 4-d intervals until mid-August. All the collected materials were fixed and stored in FAA (70 % ethanol + glacial acetic acid + formaldehyde; 90:5:5). Fruits grew rapidly until mid-June. Thus, the shells of any large fruits that were collected were removed or the ovules were directly isolated before fixing. Pistils and ovules were dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. After trimming, paraffin blocks of tissue were cut into 7 - 8 μm slices using a microtome (*Leica RM2235*, *Leica Microsystems*, Germany). Dry sections were stained with safranin-fast green and iron hematoxylin (Gao *et al*. 2010, Lin *et al*. 2010). Observations were carried out with a microscope (*Olympus BX 60*, Tokyo, Japan) and photographs were taken with the auto-photo system.

Results

Hickory is a monoecious tree with male flowers that are ternate aments and female flowers that form a terminal spike of 3 - 4 florets. The floret is naked and without perianth. At the end of April, the floret was coated by four bracts, which could be seen easily with the naked eye (Fig. 1*B*). Afterwards, the bracts unfolded gradually with the development of the florets. During early May, the stigma appeared and then green circular spots were presented (Fig. 1*C*). The pistil developed and extended outwards and, due to the different growth rate of the pistil tissues, it presented a dark-green rhombus after 2 - 3 d (Fig. 1*D*). The stigma colour changed from light red to bright red to purplish red during the developmental stages (Figs. 1*E*, 2*H*), and after it was pollinated, it quickly turned dark (Fig. 1*H*). From mid-May to the mid-June fruit size increased from 2.41 ± 0.07 mm on May 18 to 9.24± 0.08 mm on June 24 and the average growth rate was 0.18 mm per day. From June 24 to August 12, the fruits enlarged greatly and mean diameter increased to 30.3 ± 0.12 mm. After that, the fruit diameter did not increase up to harvest in early September (Figs. 1*I*,*O*).

The ovary had a chamber containing one ovule that was unitegmic and orthotropous (Fig. 2*A*). In late April, the ovule primordium was initiated in the mid-region of the ovary, and then the integument primordium began to develop. The ovules had no funiculus and single integument. However, this integument did not completely cover the micropylar end of the nucellus and the micropyle. Indeed, integument length was approximately half the length of the ovule during the mature embryo sac stage (Fig. 2*B*).

In early May, the megaspore mother cell, which was different from surrounding nucellar cells, formed in the middle of the nucellus. At this stage, stigmas of florets turned red starting from the central parts. Compared with the nucellar cells, the megaspore mother cell had a larger nucleus and denser cytoplasm (Fig. 2*C*). Dyad (Fig. 2*D*) and tetrad formed after megasporocyte meiosis. At the tetrad stage, four megaspores aligned linearly along the plane from micropylar to chalazal ends (Figs. 2*E*,*F*). Then, the megaspore at the chalazal end became a functional megaspore, while the others did not develop

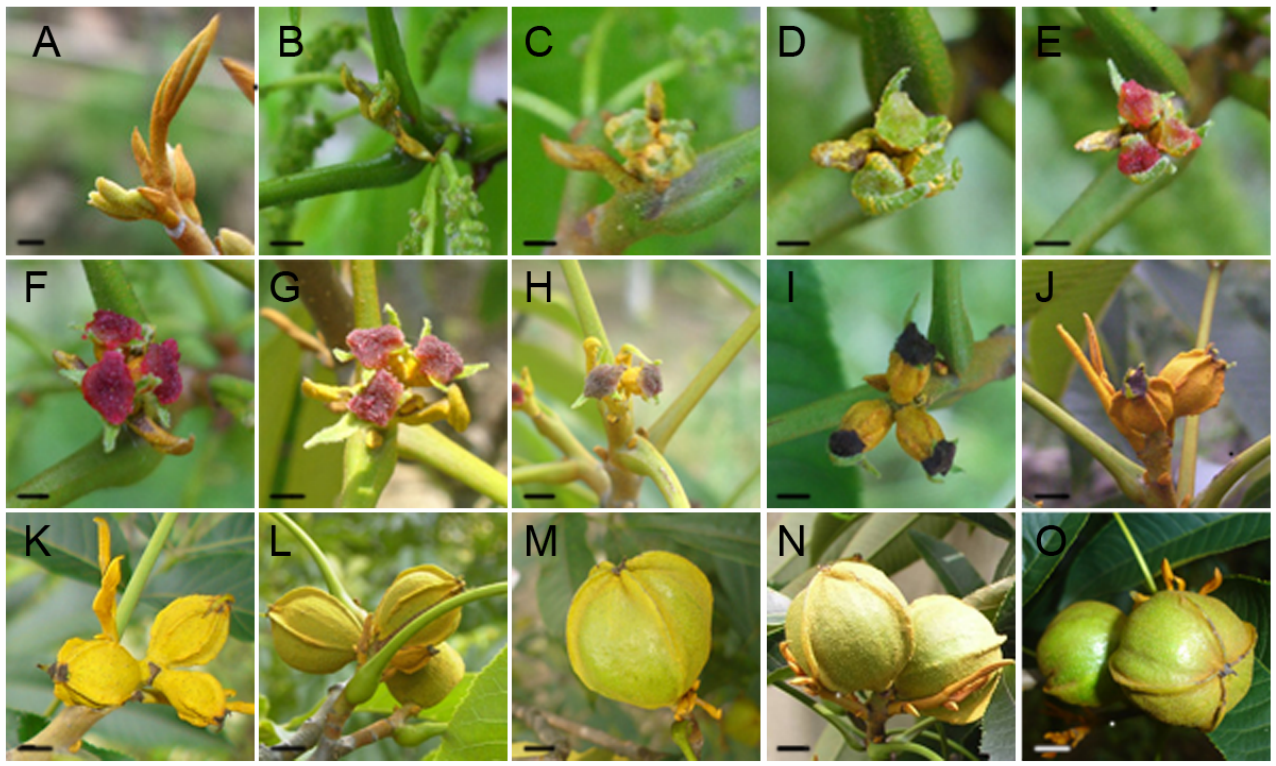

Fig. 1. Morphological observations of floret and fruit development. *A* - Female flower bud begins to differentiate at the apical end of the spring shoot at 17 April, *bar* = 0.5 mm. *B* - Florets coated with bracts become macroscopic by 4 May, *bar* = 1 mm. *C* - Female flower with three florets which are coated with four bracts at 9 May, *bar* = 6 mm. *D* - Naked florets with no perianth and rhombus-shape stigma which is not coated in bracts at 11 May, *bar* = 4 mm. *E* - Florets with light red stigmas at 13 May, *bar* = 5 mm. *F* - Florets with bright red stigmas at 16 May, *bar* = 4 mm. *G* - Florets with purplish red stigmas at 18 May, *bar* = 4 mm. *H* - Florets with dark purple stigmas at 5 DAP, *bar* = 5 mm. *I* - Young fruits at 10 DAP, *bar* = 2 mm. *J* - Young fruits at 26 DAP, *bar* = 3 mm. *K* - Young fruits at 30 DAP, *bar* = 3 mm. *L* - Young fruits at 54 DAP, *bar* = 6 mm. *M* - Young fruits at 80 DAP, *bar* = 5 mm. *N* - Young fruits at 87 DAP, *bar* = 5 mm. *O* - Young fruits at 95 DAP, *bar* = 7 mm.

and gradually degenerated to remnant vestiges (Fig. 2*F-H*). Female flowers with bright red stigmas were at the tetrad stage.

The functional megaspore continued to develop and it grew bigger (Fig. 2*H*). Concomitantly, the embryo sac enlarged. At the two-nucleate embryo sac stage, the two nuclei moved toward opposite poles along the plane between the micropylar to chalazal ends (Figs. 2*I,J*). The stigmas were purplish red on female flowers when the megagametophyte was at the four- and eight-nucleate stages. Nuclei continued to divide in the four-nucleate embryo sac (Fig. 2*K*). The eight-nucleate embryo sac had 4 nuclei at each pole, then one nucleus from each pole has migrated toward the center of the embryo sac (Fig. 2*L*). When the floret stigma was dark purple, the mature embryo sac was formed with three antipodal cells at the chalazal end and two polar nuclei and an egg apparatus at the micropylar end (Figs. 2*M-O*). The development of female flowers is asynchronous. Stigmas of florets were of different colours during the pollination period, but most were bright red and purplish red. Female flowers were mostly at the bi-nucleate or four-nucleate embryo sac stages during pollination. The embryo sac was of the typical polygonum-type.

We observed histologically that the division of the primary endosperm nucleus took place initially at 5 d after pollination (DAP; Fig 3*A*). The fusion of the two male gametes and the egg was not observed. At 10 DAP, a few free nuclei, presumably originating from the division of the primary endosperm nucleus, were detected in a vacuole-like compartment (Fig. 3*B*). These nuclei were enclosed within cytoplasmic strands, which crossed the embryo sac from the micropylar to the chalazal pole. Zygote embryos were not found at this stage. During the early stages of ovule growth, the free nuclei of the endosperm were embedded in a strand of cytoplasm along the outer margin of the central vacuole that was in contact with the nucellus (Fig. 3*E*). At 10 DAP, changes were observed for some nucellus cells above the embryo sac at the micropylar end. These cells contained a large nucleus or multiple nuclei, and these nuclei were surrounded by saccular structures (Fig. 3*C*). Furthermore, there were also changes in the cells adjacent to the sexual embryo sacs at the chalazal end, including a large

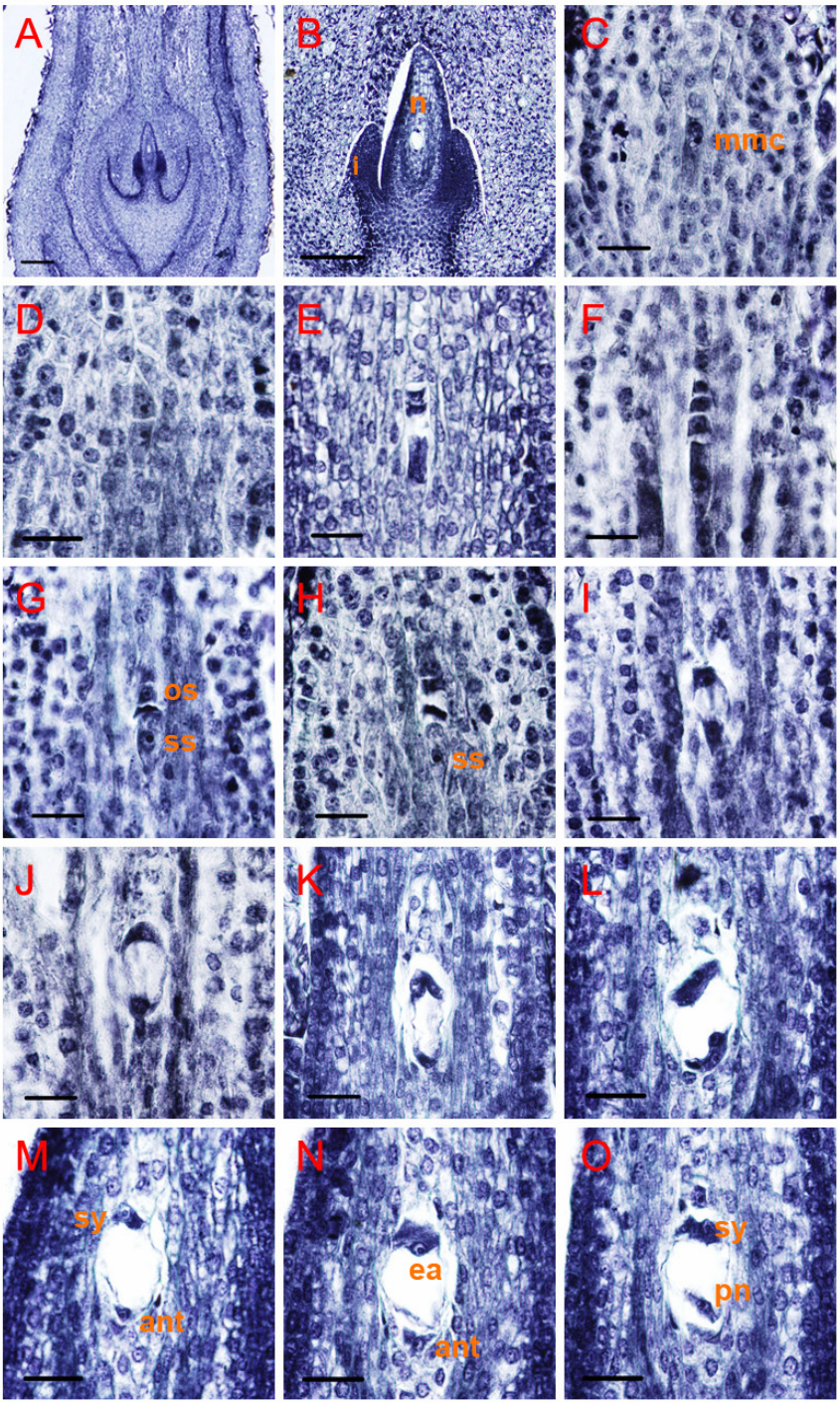

Fig. 2. Megasporogenesis and development of the female gametophyte in *Carya cathayensis*. *A* - The ovary has a chamber with an ovule that is unitegmic and orthotropous. *B* - The ovule is unitegmic, orthotropous and crassinucellate; i - integument, n - nucellus. *C* - Megaspore mother cell (MMC). *D* - Dyad. *E* - Four megaspores forming one linear tetrad. *F* - Three megaspores being dissolved at the micropylar end, while one is developing at the chalazal end. *G* - The second megaspore being dissolved at the chalazal end (os), two megaspores still being kept at the micropylar end and the one megaspore developing at the chalazal end. *H* - Three megaspores near the micropyle degenerate in succession, and the functional megaspore (ss) at the chalazal end. *I* and *J* - Bi-nucleate embryo sac with two nuclei distributed along the plane between the micropylar and chalazal ends. *K* - The four-nucleate embryo sac. *L* - The eight-nucleate embryo sac showing three antipodal cells (ant) and four nuclei at the chalazal end. *M* to *O* - Successive sections of a mature embryo sac; *M* - a synergid (sy) at the micropylar end and an antipodal (ant) at the chalazal end; *N* - the egg apparatus (ea) and two antipodals (ant); *O* - a synergid (sy) and a polar nuclei (pn). *Bars*: *A* = 200 µm; *B* = 100 µm; C - O = 20 µm.

cell nucleus and granular cytoplasm (Fig. 3*D*). These cells were isolated from the surrounding nucellar cells.

During endosperm development, the embryo sac developed gradually and the ovules enlarged. The integument grew and coated the nucellus, which mostly remained only in the nucellar beak region (Fig. 3*F*). Degradation of the nucellus was observed during the development and enlargement of the embryo sac in the ovule at 26 DAP. The nucellus had been partly absorbed from the micropylar and chalazal end regions, and nucellar cells at the chalazal end were associated with loose structures (Fig. 3*F*). Endosperm was absent from some ovules (Figs. 3*G-I*). In these ovules, the embryo sacs were narrower and nucellar embryo initials, composed of cells with differing degrees of vacuolization, were found at the edges of the embryo sacs at the chalazal end (Fig. 3*G*). Nucellar cells at the nucellar beak region showed differing degrees of vacuolization, and

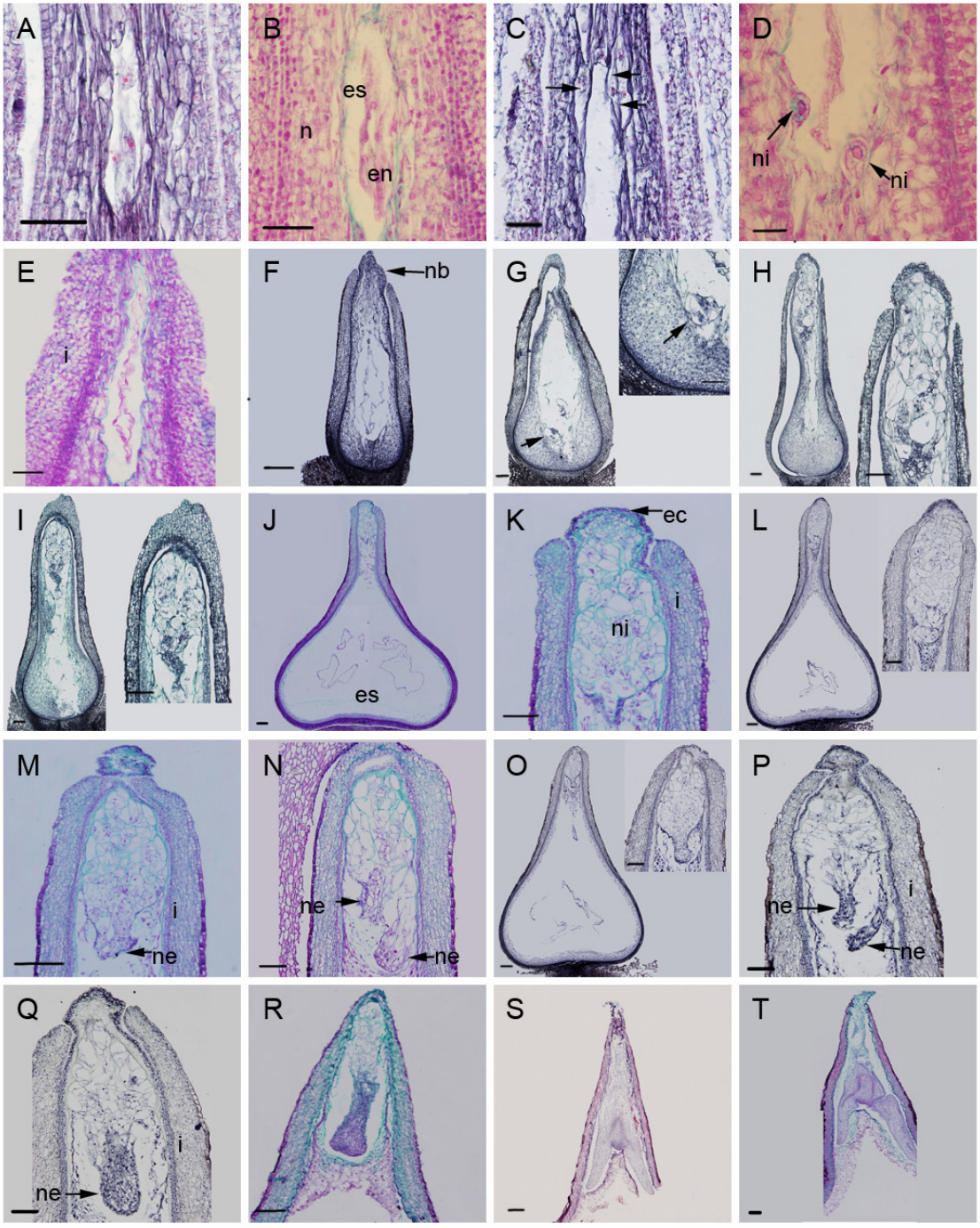

Fig. 3. Nucellar embryony and development of the nucellar embryo and endosperm. *A* - Four free nuclei divided from the primary endosperm and the egg cell at the micropylar end of the embryo sac at 5 DAP, *bar* = 50 µm. *B* - Many free nuclei were encapsulated into the annular membrane around the central vacuole at 10 DAP, *bar* = 50 µm. *C* - Some nucellar cells elongated, enlarged and contained large and/or multiple cell nuclei with saccular structures surrounding the area above the sexual embryo sac at the micropylar end at 10 DAP, *bar* = 50 µm. *D* - Nucellar embryo initials with large cell nucleus and granular cytoplasm also existed at the chalazal end of embryo sac at 10 DAP, *bar* = 50 µm. *E* - Endosperm developed and free nuclei were encapsulated into the annular membrane around the central vacuole at 19 DAP, *bar* = 50 µm. *F* - The integument coat nucellar mostly only remained at the nucellar beak region; nucellar cells were absorbed gradually at 26 DAP, *bar* = 200 µm. *G* - A nucellar embryo initial developing at the chalazal end of embryo sac in an ovule without developing endosperm at 33 DAP, *bar* = 100 µm. H and *I* - Ovules lost endosperm at 33 DAP, nucellar cells at the beak region were large and vacuolization had occurred to differing degrees; the cells formed an irregularly shaped mass nucellar embryo initial, *bar* = 100 µm. *J* - The ovule enlarged greatly, especially at the chalazal end; the nucellus has been mostly absorbed except for the epidermal cells and at the nucellar beak region during late June (38 DAP), *bar* = 100 µm. *K* - Magnified nucellar beak region observed in *J*; nucellar cells at the nucellar beak region above the embryo sac were large and contained vacuoles to differing degrees, *bar* = 200 µm. *L* - The lower magnification on the left shows that the nucellus has been mostly absorbed except for the epidermal cells and at the nucellar beak region during late June (38 DAP), *bar* = 100 µm; the magnified nucellar beak region showing large cells with vacuolization to differing degrees that formed a mass nucellar embryo initial, bar = 200 µm. *M* - A block cell mass formed above the embryo sac at the micropylar end with the development of the whole nucellar embryo initial at June 29 (42 DAP), *bar* = 200 µm. *N* - Two block-like nucellar proembryos in an ovule, *bar* = 200 µm. *O* - Lower magnification on the left shows the whole ovule at July 3 (46 DAP), *bar* = 100 µm; the nucellar beak region magnified on the right shows the rod-shaped nucellar embryo, *bar* = 200 µm. *P* - Two rod-shaped nucellar embryos in the micropylar end, of which one was initiated from the whole mass nucellar embryo initial at the nucellar beak region, while the other was initiated from the epidermal cell in the micropylar end at July 3 (46 DAP), bar = 200 µm. *Q* - Globular embryo stage similar to the zygotic embryo at 51 DAP, *bar* = 200 µm. *R* - Heart-shape embryo stage similar to the zygotic embryo at 59 DAP, *bar* = 200 µm. *S* and *T* - Cotyledon embryo stage at 65 DAP, *bar* = 200 µm (es - embryo sac; en - endosperm; i - integument; n - nucellus; ni - nucellar embryo initial; nb - nucellar beak; ec - nucellar epidermal cell; ne - nucellar embryo.

irregularly shaped embryos with dense cells were found at 33 DAP (Fig. 3*H*,*I*). This type of ovule was not found during later developmental stages.

In the ovules that contained a developing endosperm, the nucellar embryo initials were not found at the chalazal end of the embryo sac. Up to late June (38 DAP), the nucellus was absorbed, leaving mostly only epidermal cells and some nucellus at the nucellar beak region (Fig. 3*J*). From mid-June to mid-July, there was great enlargement of the ovules, especially at the chalazal end. Except for the 1 - 2 layers of epidermal cells, nucellar cells at the nucellar beak region above the embryo sac were large and formed a mass nucellar embryo initial with an obvious boundary with the surrounding cells (Figs. 3*K*,*L*). The nucellar embryo initial developed into some small compact cells. One or more cell masses formed above the embryo sac at the micropylar end during the development of the nucellar embryo initial at June 29 (42 DAP) (Fig. 3*M*,*N*). Cell masses were irregularly shaped during the early stages, and in some ovules cell masses split into two nucellar proembryos (Fig. 3*O*,*P*). Rod-shaped nucellar embryos formed during the cell division (Fig. 3*O*,*P*). The later development of the nucellar embryo was similar to the zygotic embryo and it underwent changes from globular stage (Fig. 3*Q*) to heart-shape stage (Fig. 3*R*) and, finally, to cotyledon stage (Fig. 3*S*,*T*).

Discussion

Most sporophyte reproducing plants are diploid and polyembryonic. The zygotic and apomixis embryos coexist in an ovule and can be distinguished easily according to their position in the ovule (Richards 2003). Female gametophyte development in hickory is normal, but the zygotic embryo is absent in large numbers of tissue cuttings and mature embryos all originate from the nucellus. AFLP and SSR analyses of *C. cathayensis* × *C. illinoensis* hybrids also showed that the progeny was identical with the mother (Wang *et al*. 2010). Apomixis in hickory is high and trends to be obligate, but development of a sexual embryo cannot be excluded. Most apomictic plant species are facultative for apomixis and complete obligate apomixis is rare (Asker and Jerling 1992). *Garcinia mangostana*, whose single adventitious embryo originates from an inner integument cell, is the only case of obligate apomixis in woody plants (Horn 1940). The reasons behind the absence of sexual embryos in hickory may be complex. Although germination and growth of the pollen tube on the stigma was observed, the process of pollen tubes growing through the tissue of the style and entering the embryo sac was difficult to follow with the fixatives and stains used (data not shown). In walnut, the growth of pollen tubes can proceed through either the micropyle (porogamy) or the funiculus and chalazal tissues (chalazogamy) depending on the developmental stage of the micropyle (Luza and Polito 1991). However, in this study, the single integument of hickory can not envelop the ovule in the mature embryonic sac phase. Synergids also play an important

role in the guidance of pollen tubes (Higashiyama *et al*. 2001, Higashiyama 2002). From pollen germination to fertilization, many factors may lead to fertilization failure of the egg cell.

Initial cells of nucellar embryo are evident histologically in the ovules of flowers prior to anthesis. *In vitro* culture of ovules from flowers at different prepollination stages showed that embryos could develop from ovules cultured as early as the binucleate stage of megagametogenesis in which nucellar initial cells were absent histologically (Koltunow *et al*. 1995b). Some nucellar cells showed changes in 10 DAP ovules, specifically cells were enlarged and elongated, contained a large nucleus or multiple nuclei, and these nuclei were surrounded by saccular tissues. The morphology of these cells is different from the nucellar embryo initial cells in *Citrus*. The function of these changed nucellar cells is unknown but closely related to the degradation of nucellar cells and the formation of embryogenic cells later at the nucellar beak region. The initial cells of nucellar embryo were distributed mainly above the embryo sac at the micropylar end and adjacent to the sexual embryo sac at the chalazal end. However, the mature nucellar embryo was found only at the nucellar beak region, which might be due to the inhibition of the developing endosperm as reported previously in *Citrus* (Wakana and Uemoto 1987, 1988, Koltunow 1995b). There is strong evidence that the nucellar embryo initial only existed in ovules that showed no endosperm development.

Previous studies, such as those in *Citrus*, found that all nucellar embryos originate from a single cell. Embryo initial cells first form thick walls, which isolate them from the surrounding maternal tissue, and then, in later stages, the cell walls become thinner in some of the initial cells and embryogenesis becomes asynchronous (Koltunow *et al*. 1995b). In hickory, it was shown that the nucellus was absorbed, and only several layers of epidermal cells were left, except at the nucellar beak region. Nucellar cells in this region showed morphological changes and did not have obvious boundaries between each other. Later, the whole nucellar cell mass initials formed nucellar embryos, suggesting that the nucellar embryos in hickory might not originate from just a single cell. The form of the nucellar embryony is distinct in hickory compared to that seen in *Citrus* (cluster and bud-like protuberance) (Bicknell 2004) and in *Tabebuia ochracea* (initial cell divides and forms tubular shape nucellar embryo that is composed of apical cell and basal cell) (Costa *et al*. 2004).

In many angiosperms, a 2:1 maternal:paternal ratio in the endosperm is optimal for seed development, and this results from genomic imprinting (Vinkenoog *et al*. 2003). In the vast majority (*ca*. 90 %; Mogie 1992) of apomictic plants production of viable seed depends on the presence of pollen, *i.e*., fertilization of the polar nuclei is still required for normal development of the endosperm (pseudogamy). Only a few apomictic taxa, mainly occurring in the *Asteraceae* and including genus *Taraxacum* and *Hieracium*, can develop endosperm autonomously. Moreover, *Commiphora wighti*, can form sexual and apomixis embryos simultaneously and develop endosperm autonomously (Gupta *et al*. 1996). Genomic imprinting in these autonomous apomicts is probably bypassed (Curtis and Grossniklaus 2007). Endosperm develops in most ovules and is absent in a few young fruits that later abort. This indicates that the occurrence of the nucellar embryo does not depend on endosperm development. In order to determine the source of the endosperm further, another study was conducted to bag and isolate more than 100 female flowers before the stigmas turned red. We found that young fruits dropped completely within 30 d, and it seemed that pollination was necessary for fruit setting (unpublished observation). Endosperm is triploid and this was defined basically through our preliminary study using FCSS. Thus, endosperm development is pollen dependent and endosperm formation in hickory is derived from pseudogamy. Endosperm development was essential to the nucellar embryo, although the endosperm did not supply nutrition during the early development of nucellar embryos because the endosperm was in a rapid stage of growth. Therefore, the nucellar embryo must be dependent upon the nucellus as a direct source of nutrients (Wakana and Uemoto 1987, 1988, Koltunow *et al*. 1995b). Similar to the findings in pecan (McKey 1947), the beginning of shell hardening at the micropylar end is an important turning point. Endosperm was in a rapid growth stage and the nucellus was absorbed completely in early July. The developing nucellar embryo initial cells and proembryos used the degenerated nucellus as the source of nutrition.

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