Double Fertilization and Embryogenesis of *Eustoma grandiflorum*

Qiuhong Wang¹², Yang Zhang¹, Saneyuki Kawabata³ and Yuhua Li¹*

¹College of Life Sciences, Northeast Forestry University, Harbin 150040, China
²Heilongjiang University, Harbin 150080, China
³Graduate School of Agricultural and Life Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

The double fertilization process and the development of endosperm and embryo were observed by microscopy in *Eustoma grandiflorum*. The flowers were fixed at various times after hand-pollination during stages of pollen tube extension, entry of pollen tubes into ovule, double fertilization, and early development of the embryo. Pollen grains initiated germination and penetrated the style 4 hours after pollination (HAP). Pollen tubes grew toward the ovule through the intercellular spaces along the solid style at 8 HAP. During pollen tube extension at 24 HAP, the generative cell divided into two sperm cells. Pollen tubes reached the ovary at 48 HAP, extended directly into the degenerating synergid cell and released the two sperm cells. One of the two sperm cells arrived adjacent to the egg cell at 60 HAP. Four to 5 days after pollination, a zygote was formed. The sperm nucleus adhered to the nuclear membrane of the secondary nucleus at 60 HAP. The primary endosperm nucleus was formed at 72 HAP. The zygote underwent the first mitosis 20 days after pollination. After the first divisions of the zygote, the apical cell divided transversely initially, and continued to develop to form the embryo proper; hence, the embryogenesis was of Solanad type. The mature seeds have a heart-shaped embryo. Only one spindle formed during the first mitosis of the zygote, and the fusion of male and female nucleoli took place before mitosis. The karyogamy was therefore of premitotic type.

**Key Words:** double fertilization, embryogenesis, endosperm, *Eustoma grandiflorum*, Premitotic karyogamy.

Introduction

Reproductive development of angiosperms is characterized by double fertilization, whereby one sperm cell fuses with the egg cell to produce an embryo while the second sperm cell fuses with the central cell to form the endosperm (Elizabeth and Russel, 2002; Higashiyama et al., 1997; Raghavan, 2003; Weterings and Russell, 2004). Double fertilization is represented by various dynamic processes, such as growth of the pollen tube from the stigma to the ovule, release of sperm cells in the degenerated synergid cells, migration of sperm cells to target female gametes, fusion of one sperm cell with the egg cell, and the fusion of the other sperm cell with the central cell. The entire series of events proceeds inside the pistil. Microscopic studies of these events have been conducted in many plants, such as *Lilium longiflorum* (Janson and Willemse, 1995), *Rosa rugosa* (Gudin, 1993), and *Dianthus caryophyllus* (Hoshino et al., 2000).

*Eustoma grandiflorum* (Raf.) Shinn. is a perennial herbaceous ornamental species, originated from the Southern part of north America. This species has become one of the major cut flowers due to its large and attractive flowers, long stalks, and long duration in vases (Ecker et al., 1994; Gill et al., 2000; Ohkawa, 1995; Uddin et al., 2004; Zaccai and Edri, 2002). Although all angiosperms follow double fertilization, its time course is divergent among species. The precise time course of the complete reproductive process is prerequisite information for breeding; however, such information is lacking in many horticultural crops, including *Eustoma grandiflorum*. We have previously investigated the microsporogenesis, megalosporogenesis, and development of male and female gametophytes in *E. grandiflorum* (Yang et al., 2007). In this paper, we report the process of double fertilization and the development of embryos and endosperms.

Materials and Methods

A breeding line ‘02-49’ of *E. grandiflorum* with single yellow flowers, which is maintained at Northeast Forestry University, Harbin, China was used for the
experiment. Seeds were sown on a mixture of soil:peat:sand:perlite (3:4:2:1 v/v) at pH 6.3–7.0 in containers during February 2004 and 2005 in a greenhouse at the Research Institute of Flower Biotechnology, Northeast Forestry University. The containers were enveloped in a plastic bag after sowing to prevent evaporation and placed at 20–30°C. The seeds germinated 10–30 days after sowing. In May, the young plants were acclimatized to outdoor conditions for 10–15 days and planted in well-drained soil beds, 23 cm apart, in a greenhouse at 16–29°C.

From July to September, flowers on the main shoots or the first lateral branches were hand-pollinated at 6–7 a.m. Firstly, a group of flowers were emasculated and covered with paper bags the day before anthesis. When the corolla of flowers completely unfolded, the flowers were hand-pollinated using pollen collected from other unfolded flowers of different plants. The ovaries and styles were collected at various times after hand-pollination. Eight to 10 ovaries and styles were taken at 2-hour intervals until 48 hours after pollination (HAP). Eight to 10 ovaries and styles were taken at 4-hour intervals from 48 to 96 HAP. After 96 HAP, 3–5 ovaries were taken every 2 to 3 days. The ovaries and styles were fixed in FAA [5:6:89 (v/v) mixture of formalin, acetic acid, and 70% (v/v) ethanol]. After staining with Ehrlich’s hematoxylin, the samples were embedded in paraffin and sliced into sections at a thickness of 6 to 8 μm according to Zeller (1989). The fixed sections were observed under a microscope (BX 15, Olympus, Japan).

The time course of the events during fertilization varied among embryo sacs or embryos in the ovaries. To determine the typical time courses, approximately 5,000 embryo sacs from 300 ovaries were collected and the time of the events was defined as the time when more than 70% of the observed embryo sacs or embryos were in that developmental stage.

### Results

1. **Fertilization**
   1) **Germination of pollen grains on stigma and elongation of pollen tubes**

   The type of mature 2-celled pollen grain of *E. grandiflorum* was tricoplate, having 3 germ furrows. Pollen grains adhering to the surface of the stigma initiated germination and penetration into the solid style at 4 HAP (Fig. 1c). The growth of pollen tubes directed toward the ovule was observed through the intercellular spaces along the style at 8 HAP (Fig. 1d). During the pollen tube extension (24 HAP), the generative cell divided into two sperm cells (Fig. 1e–g).

2) **Double fertilization**

   The nucellus type of *E. grandiflorum* was tenuinucellate, as reported previously (Yang et al., 2007). The outer cell layers of the ovule (integuments) enclosed the nucellus, forming a micropyle, through which pollen tubes entered the nucellus and then the embryo sac. A mature embryo sac was composed of two synergid cells (Fig. 1a), an egg cell (Fig. 1b), a central cell (Fig. 1a, b), and three antipodal cells (Fig. 1a). Pollen tubes reached the ovary and advanced along the placenta toward the micropyle at 48 HAP (Fig. 2a). A pollen tube containing two sperm cells (Fig. 2b) was observed to penetrate the micropyle. At this stage, one of the two synergid cells degenerates, and the other remains as a synergid cell. The pollen tube extended directly into the degenerated synergid cell through the filiform apparatus and released two sperm cells (Fig. 2c).

   The sperm cells migrated to the chalazal side of the synergid cells (Fig. 2d–f). One of the two sperm cells...
arrived adjacent to the egg cells (Fig. 2f) at 60 HAP and the male and female gamete cells fused subsequently (Fig. 2j, k). After the entry of the sperm nucleus (Fig. 3a), male chromatin dispersed beside the plasma membrane (Fig. 3b), while the female chromosomes remained as a form of large nucleolus in the fused egg cell (Fig. 3a, b).
b). Then the male chromatin became condensed and formed the sperm nucleolus. The sperm nucleolus enlarged gradually to the same size as the female nucleolus (Fig. 3c). The fusion of male and female nucleoli occurred 4 to 5 days after pollination, when most fused cells contained a single large nucleus, indicating the formation of a zygote (Fig. 3d).

In approximately 1% of the 5000 embryo sacs observed, both synergid cells degenerated (Fig. 2g, h), and were observed as darkly stained cells without nuclei. In some rare cases (~1%), more than two pollen tubes entered a single embryo sac (Fig. 2h, i), or two sperm

**Fig. 4.** Development of the embryo and endosperm in *Eustoma grandiflorum*. a. First mitosis of the zygocYTE at metaphase (arrow). b. Two-celled proembryo. Upper arrow refers to a basal cell at the micropylar end; lower arrow to an apical cell at the chalazal end. c. Four-celled proembryo. Each arrow refers to one of four proembryo cells. d. and e. Globular embryo. f. Heart-shaped embryo. g. The primary endosperm nucleus (arrow) at the prophase of mitosis. h. Primary endosperm nucleus at the metaphase of mitosis. Arrow refers to the metaphase plate. i. Primary endosperm nucleus at the anaphase of mitosis, two supernumerary sperm cells (arrows) in a degenerated synergid cell. j. Primary endosperm nucleus at the anaphase of mitosis. k. Two endosperm-free nuclei (arrows). l. Eight to 10 endosperm-free nuclei. m. Endosperm cells forming one layer structure. n. Two-layered endosperm cells. o. Mature endosperm. p. Mature endosperm cells. q. Degenerated endosperm cells. Scale bars, 15 μm for a to c and r to l; 30 μm for d to f; 80 μm for m to o; 150 μm for p to q.
cells were found in one degenerated synergid cell after fertilization (Fig. 2h).

The other sperm cell fused with the central cell during the same period. The sperm nucleus adhered to the nuclear membrane of the secondary nucleus after cell fusion at 60 HAP (Fig. 3a, e, f). Subsequently, the chromatin dispersed once (Fig. 3g), and the male nucleolus was formed. The size of the male nucleolus increased gradually, reaching the same size as the secondary nucleolus (Fig. 3h). After the fusion of male and female nucleoli, the primary endosperm nucleus, which could be recognized as a large nucleolus, was formed at 72 HAP (Fig. 3i).

2. Embryo development

The zygote remained dormant for about 15 days after fertilization. During this stage, the zygote elongated gradually, and its nucleus began to move towards the chalazal end. The zygote underwent the first mitosis 20 days after pollination (Fig. 4a). During this division, only one spindle was formed, indicating that the fusion of male and female nuclei was completed before the first mitosis (premitotic karyogamy). The zygote divided transversely to the long axis, forming basal and apical cells (Fig. 4b). Both the apical and basal cells divided transversely again, forming a four-celled linear pro-embryo (Fig. 4c). Then, the two cells at the chalazal end underwent longitudinal cell division into four cells (Fig. 4d). As the first division of the apical cells was transverse, the apical cell derivatives formed the embryo proper, and the basal cell contributed to form the suspensor, so the embryogenesis of *E. grandiflorum* conformed to the Solanad type. The proembryo developed to the globular stage 35 days after pollination (Fig. 4e) and became heart-shaped 60 days after pollination (Fig. 4f). Mature seeds have heart-shaped embryos.

3. Endosperm development

Unlike the embryo, the primary endosperm nucleus initiated nucleus division immediately after fertilization (Fig. 4g–k). Then, 2 free nuclei of the endosperm (Fig. 4k) formed and divided repeatedly without cell wall formation (nuclear type). More than 8–10 free nuclei formed at 80 HAP (Fig. 4l). The free nuclei continuously underwent mitosis under the central cell wall, forming coenocytic endosperm. The endosperm cell walls formed gradually from the former central cell wall to the central vacuole (Fig. 4m–o). Endosperm cells accumulated starch immediately after the completion of cellularization (Fig. 4p) and then began to degenerate, losing starch in mature seeds (Fig. 4q).

Discussion

Double fertilization is common in all angiosperms; however, there are marked variations in the types of embryo and endosperm development, the time-courses of the events during fertilization, and the types of karyogamy regarding the relationship between nuclear fusion and the cell cycle of the gametes.

The embryological characteristics of *E. grandiflorum* were in good agreement with its taxon. *E. grandiflorum* belongs to the Gentianaceae family. In Gentianaceae plants, the embryo sac is round or oblong, the ovule is anatropous, endosperm development is of the nuclear type, the pattern of cell division of the embryo is Solanad type, and the embryo seldom proceeds beyond the heart-shaped stage in mature seeds (Akhalkatsi and Wagner, 1997; Lakshminarayana and Devi, 1985).

During the fertilization process of many angiosperms, one of the synergid cells degenerates before or upon the penetration of pollen tubes into the embryo sac, the other remains as a persistent synergid cell, and only one pollen tube can enter the degenerate synergid cell. In a few embryos, it was observed that both synergid cells degenerated (Fig. 2g, h), more than two pollen tubes entered one embryo sac (Fig. 2i), or two supernumerary sperm cells co-localized in one degenerated synergid cell after fertilization (Fig. 2h). Occasionally, multiple pollen tubes in an embryo sac could cause polyspermy in flowering plants (Kapil and Bhatnagar, 1975). In *Zea mays*, additional pollen tubes could enter the embryo sac still in the proembryo phase (Möl, 1994). Among all the embryos observed in this study, however, no polyembryo could be found; therefore, eggs of *E. grandiflorum* can accept the sperm cell that arrives first.

The detailed time course of double fertilization is usually difficult to estimate in vivo, since the whole event occurs inside the ovule, which cannot be observed directly under a microscope in most flowering plants. Higashiyama et al. (1997) recorded the kinetics of fertilization in vivo by direct observation of egg apparatus using *Torenia fournieri*, in which the embryo sac is exposed from the tip of the ovule (Erdelská, 1974). The observed information can be used as milestones for understanding the fertilization process in other plants. In *Torenia*, the pollen tube arrived at the embryo sac at 9 HAP, double fertilization occurred at 10 HAP, and cell division of the embryo initiated at 28 HAP.

The estimated time course from pollen tube germination to embryo development is summarized in Table 1. The time required for each event to occur after pollination was generally later than other plants reported previously, especially than in cereal crops. The time required from pollen germination to arrival at the embryo sac was reported to be 30 min in wheat (Hoshikawa, 1959), 40 min in barley, 5 hours in *Arabidopsis* (Sandaklie-Nikolova, 2007), and 14 hours in Chinese cabbage (Liu et al., 1995), while it took 48 hours in *E. grandiflorum*. Fusion of sperm cells with egg cells was observed at 72 HAP in *E. grandiflorum*, while it took 45 min after pollination in barley (Engell, 1989), 4–5 hours in wheat, 18 hours in Chinese cabbage, and...
43–45 hours in *Cymbidium* (Nagashima, 1982). Cell division of the embryo was observed 20 days after pollination, which was much later than 22–24 HAP in barley, and 28 HAP in *Torenia*. Compared with other species of the Gentianaceae family, the time course was also later than the days after pollination in *Gentiana manshurica* (Zhu and Shen, 1989).

Nuclear fusion, or karyogamy, is the process by which the haploid nuclei of sperm and egg cells fuse to produce a single diploid nucleus. Karyogamy seems to occur within 1 to 2 hour after cell fusion (plasmogamy) in cereals, such as wheat (Tian and Shen, 1992; You and Jensen, 1985) and barley (Mogensen, 1982). In many angiosperms, karyogamy is immediately followed by mitosis (Tian and Shen, 1992). By contrast, in *E. grandiflorum*, plasmogamy occurred at 72 HAP, karyogamy occurred 4–5 days after pollination, and mitosis started 20 days after pollination.

The stage of the cell cycle during karyogamy is highly variable among seed plants (Carmichael and Friedman, 1995). In animals, karyogamy occurs at G1-phase (with DNA complement of 1C) and then the zygote progresses to S-phase and G2-phase before mitosis occurs. In angiosperms, nuclei of sperm and egg cells may undergo karyogamy in the G1, S, G2, or M-phase of the cell cycle. In *Arabidopsis*, for example, sperm cells are released at S-phase with the DNA complement of 1.4C–1.5C, and progress to G2-phase (2C) during pollen tube elongation (Friedman et al., 1999); therefore, karyogamy occurs at G2-phase of the cell cycle in male gametes of *Arabidopsis*. Gerassimova-Navashina (1960) identified three types of fertilization, i.e. premitotic, postmitotic, and intermediate. In the premitotic type, karyogamy occurs immediately following the penetration of sperm chromosomes into the egg cells and the zygote undergoes mitosis thereafter. In the postmitotic type, karyogamy does not occur immediately after plasmogamy, but replication of DNA derived from gametes occurs in the fused cell (1C–2C) before mitosis. In *E. grandiflorum*, the fused cell did not proceed to nuclear fusion immediately, but nuclear fusion occurred 4–5 days after pollination. Regarding temporal changes in the size of the sperm cell-derived nucleolus during the period between plasmogamy and karyogamy, it is likely that the cell cycle passed through the S and G2-phase during this period, suggesting G2-karyogamy. This is also supported by the fact that it usually takes a long period from the arrival of the sperm cell at the egg cell until completion of nuclear fusion in many G2-karyogamy plants.

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**Literature Cited**


