Microsporogenesis, Megasporogenesis, and the Development of Male and Female Gametophytes in *Eustoma grandiflorum*

Xinyu Yang¹, Qiuhong Wang¹² and Yuhua Li¹*

¹College of Life Sciences, Northeast Forestry University, Harbin 150040, China
²College of Life Sciences, Heilongjiang University, Harbin 150080, China

Embryological characters during microsporogenesis, megasporogenesis, and the development of male and female gametophytes in *Eustoma grandiflorum* were observed by microscopy. The results are as follows. 1. The formation of anther walls was of the dicotyledonous type. The tapetum was of the heteromorphic and glandular type. Tapetal cells on the connective side elongated radially. 2. Cytokinesis in microsporocyte meiosis was of the simultaneous type and microspore tetrads were tetrahedral. 3. Mature pollen grains were 2-celled and had 3 germ furrows. 4. The ovary was bicarpellary syncarpous and unilocular, having parietal placentas. Ovules were numerous and anatropous. 5. The archespore under the nucellar epidermis directly developed a megaspore mother cell, which in turn underwent meiotic division to form 4 megaspores arranged in a line or T-shape. The chalazal megaspore was observed to be functional. 6. The formation of the embryo sac was of the polygonum type. Before fertilization, the 2 polar nuclei fused into a secondary nucleus. The mature embryo sac was made up of 7 cells. 7. It was found at a very low rate that there were 2 megasporocytes or 2 embryo sacs in an ovule.

Key Words: *Eustoma grandiflorum*, female gametophyte, male gametophyte, megasporogenesis, microsporogenesis.

Introduction

*Eustoma grandiflorum* (Raf.) Shinn. (lisianthus, Russell prairie gentian) is a genus of the Gentianaceae family (Ohkawa, 1995). In *E. grandiflorum*, there are many cultivars producing flowers in diverse colors with various vase lives. It originated in the West Indies, Mexico, and Central and South America. Wild plants have blue-purple flowers, but intensive breeding in Japan over the past 30 years has produced cultivars with white, pink, and mauve flowers. *E. grandiflorum* has been used as a breeding material for a number of cultivars of prairie gentian (Okamoto et al., 2002). Much research in *E. grandiflorum* has been made on heredity and selection concerning floral color and shape (Jamal Uddin et al., 2002, 2004). The genetic transformation of related genes was also conducted (Ledger et al., 1997; Semeria et al., 1996).

In our laboratory, we conducted breeding experiments concerning flower traits of *E. grandiflorum* using conventional cross-breeding methods and registred several commercial cultivars, such as ‘Danlunzhusha’ (Registration Number of Heilongjiang Province in China is Heidengji2006036) and ‘Qianduixue’ (Registration Number is Heidengji2006037). At present, the breeding of cultivars adaptable to cold climate is under consideration in order to more extensively produce *E. grandiflorum* in northern China. For the introduction of cold adaptable traits, intergeneric crossing is unavoidable since cold adaptable lines are not available. The expected occurrence of cross-incompatibility will be attempted to be overcome by irradiation with a laser, which was shown to be effective in *Brassica* (Li and Yu, 2006). However, unsuccessful crossings due to events at several developmental stages such as the failure in pollen germination and pollen tube growth and the abortion of ovules and embryos will be unavoidable. Information on the development of reproductive organs in *E. grandiflorum* is essential for overcoming these. We also intend to develop an efficient breeding method of this plant by applying the floral-dip method (Bent, 2000; Clough et al., 1998; Desfeux et al., 2000), in which a more exact judgment of the developmental stage of ovules in flower buds is crucial.

The study of microsporogenesis, megasporogenesis, and the development of male and female gametophytes in *E. grandiflorum* will provide basic information for

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* Corresponding author (E-mail: lyhshen@126.com).
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Materials and Methods

The breeding line ‘02-49’ of *E. grandiflorum* with single yellow flowers was used. Seeds were sown in February 2005 in a greenhouse at the Research Institute of Flower Biotechnology, Northeast Forestry University, China. In September, more than 100 flower buds or flowers on main shoots or the first lateral branches were taken at each of seven growth stages evaluated by the length of buds, i.e., less than 0.5 cm, 0.5 to less than 1 cm, 1 to less than 1.5 cm, 1.5 to less than 2 cm, 2 to less than 2.5 cm, 2.5 to less than 3 cm, and 3 to less than 3.5 cm. The size of buds was measured in terms of the length between the top and base with the naked eye or a microscope, and that of ovaries between the stylar base and the bottom of an ovary. The anthers and ovaries were fixed in FAA (5 mL formalin: 6 mL acetic acid: 89 mL 70% (v/v) ethanol). After being stained with Ehrlich’s haematoxylin, tissues were embedded in paraffin using a conventional method, and cut at a thickness of 6 to 10 μm. Observation and photomicroscopy of sections were carried out using a microscope (BX15, Olympus, Japan).

Results

Formation of anther wall

Flowers of *E. grandiflorum* were bisexual and protandrous. Mature flowers had a calyx of 5 sepals, a corolla of 5 petals, 5 stamens attached on the corolla throat, and a single-celled ovary with 2 stigmata, similar to the results of Bailey (1950). The anthers were tetrasporangiate. At an early stage of development, archesporial cells, which were recognizable by their large volume and conspicuous nuclei, differentiated below the epidermis of anthers (Fig. 1a). These cells divided periclinally to form outer primary parietal cells and inner primary sporogenous cells (Fig. 1b). The primary parietal cells divided periclinally and anticlinally to form 2 layers of secondary parietal cells (Fig. 1c). The outer secondary parietal cells formed a subepidermal endothecium and two middle layers by periclinal and anticlinal divisions (Fig. 1d, e). The middle layers had a common histogenetic origin with the endothecium (Fig. 1d, e). The inner secondary parietal cells gave rise to the tapetum, which partly originated from the ground tissue near the connective tissue (Fig. 1f, g). Thus, the tapetum was of dual origin. The microsporangial wall formation was therefore of the dicotyledonous type. The endothecium comprised 1 layer. The middle layers appeared to undergo further divisions to form 3 layers. The anther wall prior to maturation was usually comprised of 6 layers, i.e., an epidermis, an endothecium, 3 middle layers, and a layered tapetum (Fig. 1e–g).

Cells of the tapetum on the connective side showed radial elongation and intruded into the anther locule to form ‘placentoids’, which were named by Steffen and Landmann (1958) (Fig. 1g). Tapetal cells were unineculeate with 2 nucleoli, each of which showed a round or ellipsoidal nucleus with a dense cytoplasm throughout development (Fig. 1e–g). At about the time of meiosis of a microspore mother cell, tapetal cells showed indistinct walls and appeared to be degenerating (Fig. 1h). At the stage of one-nucleate free microspores and two-nucleate pollen grains, tapetal cells degenerated completely. The tapetal cells degenerated at their original sites. Therefore, the tapetum was of the heteromorphic, named firstly by Bhojwani and Bhatnagar (1979), and glandular type.

Microsporogenesis and microgametogenesis

Simultaneously with changes taking place in the wall of the microsporangia, the primary sporogenous cells underwent mitosis to form secondary sporogenous cells, from which microsporocytes were derived (Fig. 1i). Microsporocytes were recognizable by their large volume, dense cytoplasm, and conspicuous nuclei. The microsporocyte underwent meiosis, and the process of meiosis involved 2 cell divisions. Meiosis I includes the prophase, which is sub-staged as leptotene (Fig. 1j), amphitene, pachytene, diplotene, and diakinesis (Fig. 1k), metaphase (Fig. 1l), anaphase, and telophase (Fig. 1m). As a result, a microspore dyad is formed. A microspore tetrad was formed via the prophase, metaphase (Fig. 1n, o), anaphase, and telophase. There were 2 kinds of microspore tetrads: tetrahedral tetrads occupying 95% of observed microspore tetrads (Fig. 1p, q) and decussate tetrads at 5% (Fig. 1q). There was a thick callous wall outside a microspore tetrad (Fig. 1p). As the microspore developed, callous walls disappeared (Fig. 1q, r). During meiosis, cytokinesis was of the simultaneous type (Fig. 1m–r).

Free unineculeate microspores were separated from the tetrad. Each microspore had a dense cytoplasm, conspicuous wall, and a prominent and centrally placed nucleus (Fig. 1s). As the central vacuole developed, the nucleus took a peripheral position (Fig. 1t). At this stage, the microspore was uninucleate and peripheral. Mitosis (Fig. 1u) of the microspore nucleus resulted in the formation of 2 unequal cells, i.e., a larger vegetative and a smaller generative one (Fig. 1v). Then, the generative cell was submerged in the cytoplasm of a vegetative cell (Fig. 1x). Mature pollen grains were two-celled (Fig. 1x).

Megasporogenesis and megagametogenesis

There were approximately 2000 to 3000 ovules in 4 rows in the placenta of an ovary in *E. grandiflorum*. The ovary was superior, bicarpellate, and unilocular with parietal placentae. The integument was initiated by periclinal and oblique divisions at the base of the nucellus. The ovule was unitegumental. The integument reached the top of the nucellus and formed a micropyle by continued division. Ovules in *E. grandiflorum*
Fig. 1. Formation of the anther wall, microsporogenesis, and microgametogenesis in *Eustoma grandiflorum*. a. Archesporial cells. The arrow indicates an archesporial cell. b. Periclinal division of archesporial cells. c. Sporogenous cells and secondary parietal cells beneath the epiderm. d. The outermost secondary parietal cells divided the endothecium (end) and middle layer (ml), showing the dicotyledonous type of wall formation. The arrows refer to the four layers from the left to right: the epiderm (epi), endothecium (end), and two middle layers (ml). e and f. Anther wall composed of the epidermis (epi), endothecium (end), middle layer (ml), and tapetum (tap). The arrows refer to the six layers from the left to right: the epiderm (epi), endothecium (end), three middle layers (ml), and the tapetum (tap). The microspores (mic) are in the middle. g. Heteromorphic and glandular tapetum. The arrows delineate the tapetum (tap) on the two sides. h. Degenerated tapetal cells. The arrow shows a degenerated tapetal cell (tap). i. Microspore mother cells (mmc). The arrow refers to the tepetal cell (tap) and a microspore mother cell (mmc). j. Meiosis of the microsporocyte, leptotene in prophase. k. Diakinesis in prophase. l. Metaphase. m. Telophase. n and o. Metaphase. p and q. Tetrahedral and decussate tetrad. The arrow refers to the thick callous wall outside a microspore tetrad. r. Degenerated callous wall. s. Free uninucleate microspores. t. Uninucleate peripheral stage of microspore. u. Telophase of microspore mitosis. v. Vegetative cells and generative cells. The arrows refer to the vegetative cell and generative cell. w. The generative cell was submerged in the cytoplasm of large vegetative cells. The arrows refer to the generative cell and the nucleus of the vegetative cell. x. Two-celled pollen grains. The arrows refer to the generative cell and the nucleus of the vegetative cell. Scale bar=15 µm for a to f and i, 80 µm for g and h, 10 µm for j to x.
Fig. 2. Megasporogenesis and megagametogenesis in *Eustoma grandiflorum*. a. Single archesporial cells as a megasporocyte, tenuinucellate. The arrow refers to the archesporial cell. b. Meiosis of the megasporocyte, leptotene in prophase. c. Amphiote in prophase. d. Diakinesis in prophase. e. Metaphase. f. Anaphase. g. Prophase. h and i. Metaphase. j. Linear tetrad of megaspores. The arrows indicate the three degenerated micropylar megaspores (dm) and a functional megaspore (fm) at the chalazal end. k. Three degenerated micropylar megaspores of a tetrad (dm), and the functional megaspore (fm) at the chalazal end. The arrows refer to the three degenerated micropylar megaspores (dm) and a functional megaspore (fm) at the chalazal end. l and m. A bi-nucleate embryo sac. n. Four-nucleate embryo sac. o. Five nuclei of an eight-nucleate embryo sac. The arrows refer to five nuclei of an eight-nucleate embryo sac. p. Two polar nuclei (pn). The arrows refer to the two polar nuclei (pn). q and r. Three micropylar nuclei became the egg (en: egg nucleus) and 2 synerids (sy), and comprised the egg apparatus. The chalazal nuclei became 3 antipodals (ant). The polar nuclei fused at the center and the resulting fusion moved close to the egg apparatus. The arrow indicates the egg nucleus (en). s. 2 archesporial cells. t. 2 embryo sacs. Scale bar = 10 µm for a to k, p, q and s, 20 µm for l to o, r and t.
were anatropous.

A single archesporial cell (Fig. 2a) differentiated under one-layered epidermal cells in the young nucellus. This archesporial cell functioned directly as the megasporocyte, which was characterized by a large nucleus and dense cytoplasm. Thus, the ovule was tenuinucellate (Fig. 2b–d). It was found at a low rate that there were 2 archesporial cells in an ovule (Fig. 2s).

The megasporocyte underwent meiosis to form a megaspore dyad. Meiosis I includes the prophase, which is sub-staged as leptotene (Fig. 2b), amphitene (Fig. 2c), pachytene, diplotene, and diakinesis (Fig. 2d), metaphase (Fig. 2e), anaphase (Fig. 2f), and telophase. Subsequently a megaspore tetrad was formed via the prophase (Fig. 2g), metaphase (Fig. 2h, i), anaphase, and telophase. Finally, these processes gave rise to a linear tetrad of meiosis I of the pachytene, diplotene, and diakinasis (Fig. 2d), metaphase (Fig. 2e), anaphase, and telophase. Thus, the mode of embryo sac formation was of the polygonum type. Three micropylar megaspores (Fig. 2j). While 3 micropylar megaspores of the tetrad eventually degenerated, the chalazal one became functional (Fig. 2k). The functional megaspore developed successively into a two- (Fig. 2l, m), four- (Fig. 2n), and eight-nucleate embryo sac (Fig. 2o) by 3 meiotic divisions. Thus, the mode of embryo sac development was the same as those reported for other species of Gentianaceae in terms of the glandular tapetum, simultaneous occurrence of microsporocyte meiosis, two-celled pollen grains, the tenuinucellate, unitegmen-tal, and anatropous ovules, and the polygonum-type embryo sac (Bhojwani and Bhatnagar, 1997; Chen et al., 2000a, b; Davis, 1966; Ho and Liu, 1999; Ho et al., 2000; Johri, 1992; Liu and Ho, 1996, 1997; Xue et al., 1999, 2002a, b; Xue and Li, 2005; Zhu and Shen, 1989).

The tapetum during development of the anther wall was of the heteromorphic and glandular type. This type of tapetum was reported for some plants in Gentianaceae, in which tapetal cells on the connective side elongated radially and intruded into anther locules to form ‘placentoids’ (Chen et al., 2000a, b; Davis, 1966; Ho and Liu, 1996, 1997; Xue et al., 1999, 2002a, b; Xue and Li, 2005; Zhu and Shen, 1989), so the tapetal cells showed dimorphism (Ho and Liu, 1999). At about the time of pollen tetrads, the walls of the tapetal cells became indistinct and the tapetal cells degenerated at their original site. The tapetal cells degenerated completely at the stage of 1-nucleate pollen grains and the nucleus near the wall. Thus, the tapetum is heteromorphic and glandular. In view of its structure and function, this kind of tapetum is considered to be more evolutionally advanced than the normal glandular type (Zhu and Shen, 1989).

The relationships between the bud length and developmental stage shown in Table 1 can be used in the research of embryogenesis and relative molecular embryology in *E. grandiflorum*. During genetic transformation by the floral dip method, the ovules are the target cells, and *Agrobacterium tumefaciens* enters the ovary before the ovules seal (Bent, 2000). This means

### Table 1. The relationship between the size of flower buds or flowers and the development of pistils or stamens in *E. grandiflorum.*

<table>
<thead>
<tr>
<th>Length of flower buds and an ovary</th>
<th>Developmental event in pistils</th>
<th>Developmental event in stamens</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2–0.3 cm bud</td>
<td>Ovule primordium</td>
<td>Archesporium</td>
</tr>
<tr>
<td>0.6–0.7 cm bud</td>
<td>Ovary primordium</td>
<td>Microspore mother cells</td>
</tr>
<tr>
<td>0.7–1 cm bud and 0.3 cm ovary</td>
<td>Archesporium</td>
<td>Microspore tetrads</td>
</tr>
<tr>
<td>1–1.6 cm bud and 0.3–0.5 cm ovary</td>
<td>Meiosis of megasporocytes</td>
<td>Mononuclear microspores</td>
</tr>
<tr>
<td>1.8–2.6 cm bud and 0.6–0.8 cm ovary</td>
<td>Development of embryo sacs</td>
<td>Two-celled pollen</td>
</tr>
<tr>
<td>3.5 cm bud and 1.0–1.2 cm ovary at anthesis</td>
<td>Mature embryo sacs</td>
<td>Pollen grains released</td>
</tr>
</tbody>
</table>

Discussion

The method of embryogenesis in *E. grandiflorum* was the same as those reported for other species of Gentianaceae in terms of the glandular tapetum, simultaneous occurrence of microsporocyte meiosis, two-celled pollen grains, the tenuinucellate, unitegmen-tal, and anatropous ovules, and the polygonum-type embryo sac (Bhojwani and Bhatnagar, 1997; Chen et al., 2000a, b; Davis, 1966; Ho and Liu, 1999; Ho et al., 2000; Johri, 1992; Liu and Ho, 1996, 1997; Xue et al., 1999, 2002a, b; Xue and Li, 2005; Zhu and Shen, 1989).
that successful transformation relates closely with the morphological structure of the floral organ. It is suggested that the most appropriate time for transformation by the floral dip method should be when the length of a bud is shorter than 1 cm or that of an ovary is less than 0.3 cm. The developmental event in the pistil at this time is the archesporium, while the integument has not yet been formed. Thus, the archesporium with only one-layer of nuccellar cells outside maybe as good as naked in the ovary, so that Agrobacterium tumefaciens will be able to enter easily.

During megasporogenesis and megalgametogenesis, cases in which an ovule had 2 archesporial cells (1 in about 900 ovules), 2 megaspores (2 in about 1100 ovules), 2 functional megaspores (5 in about 1300 ovules), or 2 embryo sacs (2 in about 1700 ovules) were rarely observed. For an ovule having 2 embryo sacs, each embryo sac was normal, i.e., it was composed of 7 cells and had an egg cell. Since the occurrence of these 2 embryo sacs in 1 ovule is probably caused by various kinds of factors inside and outside of the cells, its cytological mechanism should be further clarified in the future.

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Literature Cited


