The Growth of Pollen and Female Reproductive Organs of Ginkgo between Pollination and Fertilization

Yoshinori Nakao*, Kenji Kawase**, Shuji Shiozaki, Tsuneo Ogata and Shosaku Horiuchi

College of Agriculture, Osaka Prefecture University, Sakai, Osaka 599–8531

Summary

Approximately 120 days passes from pollination until fertilization in *Ginkgo biloba*. After pollination in late April or early May, the pollen develops in the pollen chamber until fertilization. The generative cell in the pollen grain divides into two cells; one differentiates into a spermatogenous cell, the other into a sterile one. The spermatogenous cell then divides into two sacs, separated by a membrane; these sacs, in turn, differentiate into sperms that swim to the egg to affect fertilization in early September.

With the enlargement of the endosperm, the nucellus, enveloping it at the bottom of the pollen chamber, cracks. The crack is formed concomitantly with the development of a protuberance which is produced on top of the endosperm. The growth of the keeps pace, allowing the sperm to swim to the egg.

Key Words: fertilization, *Ginkgo biloba*, ovule, protuberance on the endosperm, sperm.

Introduction

Ginkgo, *Ginkgo biloba*, is the sole living survivor of the phylum *Ginkgophyta*. The large number of ginkgo fossils found in a stratum of the Paleozoic era, indicates that they were the most thriving gymnosperm in the Jurassic Period. All *Ginkgo* spp., except *G. biloba* were exterminated during the glacial period so that no wild *G. biloba* exists. However, many ginkgo trees have been preserved and cultivated for food or as ornamental trees in the temperate zone. The commercial cultivation area of ginkgo in Japan has gradually increased in the last 20 years, because of the species value as health food and medicinal source.

The egg of ginkgo is fertilized by the free-swimming sperm, similar to the ferns and algae (Hirase, 1896). Lee (1955) and Friedman (1987a) reported that the ginkgo pollen grains, produced in the male gametophyte, are transferred to the female gametophyte by wind; and the pollen enters the ovule through a micropyle together with the pollination droplet secreted from the female gametophyte. The pollen grain produces a pollen tube with a highly branched structure, which penetrates into the wall of the pollen chamber (Friedman, 1987a), and keeps growing for several months. Late in ovule development, the sperm, which differentiates from the spermatogenous cell (SPC) of the pollen grains, swims

to the egg and fertilizes it. There are a few reports on the relationship between the ovule and the development of pollen grains during the ovule development period and how the sperm moves to the egg in the archegonium despite the absence of a route at the bottom of the pollen chamber at the time of pollination. We observed the behavior of pollen grains in detail with a microscope during the period from pollination until fertilization.

Materials and methods

Studies were conducted in 1994. Pollen grains and ovules were collected from about 25-year-old ginkgo trees growing in an ornamental plantation of Osaka Prefecture University, Sakai, Osaka.

Observation of pollen by light microscopy

Pollen grains obtained from anthers in late April and early May were fixed in acetic acid and ethanol (1:3, v/v) solution for more than 24 hr. Then, they were observed with a light microscope after staining with 0.01% gentian violet.

Observation on the development of ovule and pollen in vivo

Ovules were fixed and stored in a phosphate buffer (pH 7.4) containing 3% glutaraldehyde and 1% para-formaldehyde at 4 °C for more than 24 hr. Ovules were cut into thin sections, which were washed in phosphate buffer for more than 6 hr, and then dehydrated in an ethanol series by soaking in: 1) ethanol: methanol: diethyl ether (2: 1: 2, v/v/v) for 12 hr; 2) ethanol: diethyl ether (1: 1, v/v) for 24 hr; and 3) ethanol: diethyl ether (1: 1, v/v) containing 1%-celloidin for 2–4 days

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*Present address: The Institute of Enology and Viticulture, Yamanashi University, Kitashin, Kofu, Yamanashi 400–0005

**Present address: Fruit Tree Research Institute, Kumamoto Agricultural Research Center. Shimomashiki, Kumamoto 869–0524

depending on the sample size. The celloidin solution was concentrated in the glass chamber for a few days and hardened with chloroform vapor. The samples in celloidin resin (1mm thick) were cut and kept in isobutyl alcohol for 12 hr, then immersed in benzene for 5 hr. They were then put into a mixture of paraffin and benzene (1: 1, v/v) in an incubator at 58 °C and the paraffin was concentrated by evaporating the benzene. Finally, the samples were left to stand for 3 days to allow complete permeation by paraffin.

Sections of the ovules from 12 μm to 20 μm thick, were prepared with a microtome. The sections were stained with saffranin and fastgreen after removal of the paraffin and were observed under a light microscope.

Observation of hardening of meso-integument

Ovules were collected on June 14, July 1, 15, and 22. They were halved longitudinally, and the halves treated with 1% phloroglucinol: 95% ethanol and 12 N HCl

Fig. 1. Pollen grain under a dry condition, fixed with ethanol-acetic acid (3:1) and stained with gentian violet. Bar = 10 μm .

which stained lignin to dark red.

Observation of the pollen in ovules after meso-integument hardening

After peeling off the outer-integument and meso-integument, 5mm squares of nucellus around the pollen chamber on the endosperm were excised. The pollen grains on the inside of the nucellus were observed under a stereoscopic or light microscope.

Results

The pollen grains during the pollination period were 25–30 μm in the major transverse axis and 15–20 μm in the major conjugate axis; they were looked boat-like in shape with a germination groove along the axis (Fig. 1).

Fig. 5. A pollen tube which did not penetrate into the wall. It developed a few branches. Bar = 200 μm . TC; Tube cell. WP; Wall of pollen chamber.

Fig. 2–4. Thin sections of ovule stained with saffranin and fastgreen from early May to mid July. Arrows show pollen grains. Bar = 100 μm . E; Endosperm. I; Integument. NU; Nucellus. Fig. 2. Two pollen grains in micropyle. Fig. 3. The pollen grain entered the pollen chamber. Fig. 4. Five pollen grains growing in the pollen chamber.

Fig. 6. Developing pollen grain in a pollen chamber in mid-July. A, An unstained spermatogenous cell 100 μm in diameter, in a tube cell. Bar = 50 μm . PC; Prothallial cell. SC; Sterile cell. SPC; Spermatogenous cell. N; Nucleus. TC; Tube cell. B, Spermatogenous cell stained with acetocarmine solution; SPC is stained deeply.

Fig. 7. Pollen grains in the pollen chamber in mid-August. Bar = 200 μm .

Fig. 8. A spermatogenous cell divided into two cells by the membrane. Arrow shows spirally arranged cilia. Bar = 50 μm . Two sperm cells with moving cilia (right). MB; Membrane. S; Sperm.

The spherical pollen grains entered the pollen chamber with the pollination droplet through a micropyle in early May (Fig. 2, 3) and remained there until in mid-July (Fig. 4). The pollen grains were fixed onto the wall of the pollen chamber by their pollen tubes which penetrated into them, but a few pollen tubes grew out of the wall (Fig. 5).

Within the pollen, the generative cell (GC) divided into two cells. One of them differentiated into a spher-

ical spermatogenous cell (SPC), 100 μm in diameter with a big nucleus at the center (Fig. 6A). Another cell changed into a sterile cell (SC), while a prothallial cell (PC) elongated toward the SPC through the center of the SC, and the PC was surrounded by SC. The SPC was deeply stained with acetocarmine (Fig. 6B). An elliptical nucleus stained less intensely than did the cytoplasm in SPC. At this stage, the pollen grains were 200 μm diameter (Fig. 7). Subsequently, the SPC, which divided equally into two sacs separated by a membrane (Fig. 8), differentiated into two sperms. Initially, the cilium propelled the sperm in SPC, allowing it to break its the membrane and to swim in the tube cell. The sperm emerged from the PC at the top of the pollen grain (Fig. 9). The spherical sperm, approximately 60 μm in diam-

Fig. 9. A, Sperm swimming in the pollen tube. Bar = 50 μ m. WP; Wall of pollen chamber. S; Sperm. B, A sperm leaving the pollen tube near prothallial cell. C, Sperm swimming outside the pollen grain.

eter, swam with its cilium arranged spirally along its top (Fig. 10). The sperm swam from the pollen chamber to fertilize an egg located at the top of the endosperm (Fig. 11, 12).

Hardening of the meso-integument started from the top of the ovule early in July and extended toward the stalk, but stopped short of the micropyle (Fig. 13). Around August 10, the outer integuments of the ovules were green and immature, but the meso-integument had hardened, as the ovules did on September 18 (Fig. 14).

A protuberance developed at the top of the endosperm

Fig. 10. Sperm of ginkgo swimming. Arrow shows forward direction. Bar = 20 μ m.

Fig. 11. Sperm swimming to egg at the top side of the endosperm. Bar = 500 μ m. EG; Egg cell. E; Endosperm. S; Sperm.

Fig. 12. A schematic drawing of a vertical section around the top of the ovule. Arrows indicate pathway for pollination and the fertilization. EG; Egg cell. E; Endosperm. I; Integument. NU; Nucellus. P; Protuberance. S; Sperm.

Fig. 13. Onset of hardening of the meso-integument near the top of the ovule. Lignin which accompanies the hardening is stained dark red by phloroglucinol and HCl. Arrow shows the hardening of the meso-integument. A; June 14. B; July 1. C; July 15. D; July 22.

Fig. 14. Vertical section of two ginkgo ovules with the hardened the meso-integument. Right: Immature ovules with green and hard outer integument on August 10. Left: Matured ovule with the yellow and soft outer integument on September 18.

Fig. 15. A, B, and C are sections through a protuberance at the top of the endosperm stained with saffranin and fast-green. NU; Nucellus. EG; Egg cell. E; Endosperm. P; Protuberance. D is a section observed under a stereoscopic microscope. A; August 20. B; September 2. C and D; September 9. Bar = 500 μ m.

as it formed with the eggs within (Fig. 15). The protuberance grew and elongated, breaking the bottom of the pollen chamber synchronized with involution of the nucellus.

Discussion

The dry pollen grain of ginkgo has a boat-like shape (Sahashi and Ueno, 1986), but it placed on an agar

medium or in a highly humid condition, it enlarges and divides into four cells: TC, GC, and two PCs (Friedman and Gifford, 1988; Friedman, 1987b; Gifford and Larson, 1980; Laurain et al., 1993). Pollen grains placed on an agar medium at 25 °C germinated 3 or 4 days later (Rohr, 1980; Nakao et al., 1998). Pollen grains are carried by wind from anthers on catkins on a male tree to an ovule on a female tree which they enter through the micropyle, aided by a drop of liquid secreted at the top of the ovule. Ginkgo does not have the double fertilization system as in angiosperms. Instead, the genome of endosperm remains $n=12$ even after fertilization (Wang and Chen, 1983). In our observation, the pollen tube of ginkgo did not elongate and transport the sperm nucleus to an ovule for fertilization but became fixed on the wall of the pollen chamber to absorb nourishment for growth.

The pollen tube in angiosperm elongates rapidly and reaches the ovule (Kitajima et al., 1993; Nakamura, 1994; Shuraki and Sedgley, 1994; Wang and Horiuchi, 1990), whereas the pollen grains of ginkgo are carried into a pollen chamber together with the pollination droplet. Our observation revealed that some pollen grains remained in the pollen chamber without enlargement until mid-June. We suppose that such pollen grains do not grow or grow slowly so that they could enter the pollen chamber with the secretion of a pollination droplet. They remain in the pollen chamber as long as 4 months before dividing in vivo. In vitro, however, they do not divide normally so that sperms are not formed, whereas some pollen grains grow abnormally (Tulecke, 1957).

Hardening of the meso-integument of ginkgo started from the top of the ovule, except around the micropyle, and extended to the end of the ovule stalk. In ginkgo, the hardening along the micropyle is late because of the late fertilization, which was supposed to keep the humidity in the pollen chamber high and promote the growth of pollen grains.

There have been no reports on the formation of a path for sperm to fertilize the eggs. We hypothesize that as the nucellus degenerates, its contents are absorbed by the enlarging endosperm. Concomitantly, a protuberance at the top of the endosperm enlarges along with the egg development. This protuberance cracks the bottom of the pollen chamber, where the nucellus is thin, providing an entrance for the ciliated sperm to enter and swim to an egg and fertilize it.

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イチョウの受粉から受精期までの花粉および雌性生殖器官の生長

中尾義則*・河瀬憲次**・塩崎修志・尾形凡生・堀内昭作

大阪府立大学農学部 599-8531 堺市学園町1-1

摘 要

イチョウの受粉は4月下旬から5月上旬に風媒により行われ、胚珠に到達した花粉は受粉滴とともに花粉室内に引き込まれ生長する。花粉は始め4細胞から成り、その中の生殖細胞が2個に分裂し、その一つは精原細胞となった。精原細胞の分裂により形成された精子は花粉管から自ら泳ぎ出て、胚乳頂部の造卵器内に形成された卵と9月上旬に受精した。この間、受粉から受精までに約120日間を要した。

受粉時、花粉室内と卵とをつなぐ通り道は存在しなかった。しかしながら、胚乳の発達に伴い花粉室の底部が押し広げら

れると同時期に珠心が退化して亀裂を生じた。また、同時に形成される胚乳の頂部の突起が発達し、花粉室底部の亀裂形成の補助をするとともに受精時までの花粉室の空間を確保することで受精をより確実なものにしていると考えられる。

* 現在: 山梨大学工学部附属ワイン科学研究センター 400-0005
山梨県甲府市北新

** 現在: 熊本県農業研究センター 869-0524 熊本県下益城郡
松橋町