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Fertilization in *Brassica campestris* ssp. *pekinensis* and its duration of each stage

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Abstract This paper reports the process of fertilization in *Brassica campestris* ssp. *pekinensis* and the duration of each stage. The results are as follows: (1) Pollen germinates on stigma 2–3 h after pollination. (2) 4–8 h after pollination, pollen tube grows in the style. (3) 8–14 h after pollination, pollen tube grows in the ovary and gets into the ovule via the micropyle. (4) 16 h after pollination, one sperm nucleus moves to the egg and enters it. (5) The sperm nucleus adheres to the nuclear membrane of the egg 18 h after pollination. (6) 20 h after pollination, it enters the egg nucleus and male chromatin gradually disperses and 24 h after pollination, a male nucleolus appears. A large female nucleolus and a small male nucleolus occur in the nucleus of the fertilized egg, and zygote formed. The dispersing of sperm chromatin in the egg nucleus takes about 4 h. (7) 32–34 h after pollination, the division of zygote begins. The dormancy stage of the zygote lasts for about 8–10 h. (8) The pair polar nuclei lie in the chalazal end of the egg before fertilization, which may fuse into a secondary nucleus or not. (9) 16–18 h after pollination, the sperm nucleus moves to the polar nuclei or the secondary nucleus. 18 h after pollination, the sperm nucleus adheres to the nuclear membrane of the polar nuclei or that of the secondary nucleus. (10) 20 h after pollination, the sperm nucleus enters one of the polar nuclei or the secondary nucleus and a triple fusion takes place. The process of fusion is similar to the karyogamy but faster. The dispersing of the sperm chromatin in the polar nucleus or secondary nucleus takes about 2 h. (11) 22 h after pollination, the primary endosperm nucleus formed. The female and male nucleoli cannot fuse with each other before mitotic division of the primary endosperm nucleus. (12) 24 h after pollination, the division of the primary endosperm nucleus actually takes place.

Keywords *Brassica campestris* ssp. *pekinensis*, fertilization

1 Introduction

Studies on Chinese cabbages (*Brassica campestris* ssp. *pekinensis*) have greater emphasis on cultivation and breeding. In the field of reproduction biology, the researches mainly rested on the life history (Chen, 1984), flower bud formation, anthesis characteristics (Li, 1964; Wu, 1979; Ma et al., 1986), pollination and fertilization process of self incompatible line Chinese cabbages (Ma et al., 1986; Zhang et al., 1991), cell morphology of anther and pollen development (Wang et al., 2001a), male sterility or male abortion and embryogenesis (Wang et al., 2001b; Ma and Zheng, 1992), etc. However, the achievements in the research of the fertilization process of Chinese cabbages as well as the duration of each stage are seldom reported. This paper's contribution is not only to reinforce the basic biological theory in the fertilization of Chinese cabbages but also to design a relative schedule for plant transformation through pollen-tube pathway method.

2 Materials and methods

Habai2-1, Habai3-2, and Huaibai6 were selected as experimental materials. Before sowing, seeds were soaked until germination and then were vernalized for 1–1.5 months under 0°C–4°C temperature. They were transplanted to the garden of the Biology Department of Harbin Normal University in mid-June, 2001, early June, 2002, and mid-May, 2003, respectively. The gynoecea after pollination were fixed on the following dates, from August 3 to 15, 2001, July 13 to August 15, 2002, and June 18 to July 3, 2003, respectively and at 6 on the following day they began to receive artificial cross pollination with the pollination time recorded. Within 72 h after pollination, the gynoecea were fixed once every other hour (80 gynoecea at a time), with Kano—a stationary liquid (glacial acetic acid: anhydrous alcohol = 1:3). 2 h after fixation, they were preserved in 70% ethanol in the refrigerator, then

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stained wholly in Ehrlich's haematoxylin for 2 days, and then sliced using the Ozachromium method at 8 μm thickness, and observed and photographed under an optical microscope (Olympus BH-2).

3 Results and analyses

3.1 Germination of pollen grains on stigma and growth of pollen tubes

The mature pollen grains of Chinese cabbages were viewed as a three cell morph with one vegetative cell and two sperm cells (Plate I-1). The sperm cell showed a long oval and the stigma was trunk type with its surface covered by a layer of well-developed papillary cells (Plate I-2). Inside the style there were some thin parietal cells protruding vertically, which were viewed as the transmitting tissue (Plate I-3). The ovary consists of two carpels, where, on the edge of adnation grows out a pseudoseptum made of thin parietal cells, separating the ovary into two chambers. In the pseudoseptum, thin parietal cells were protruding vertically—the transmitting tissue was connected with that of the style.

Two hours after pollination, pollens began to germinate on the stigma and the pollen tube grew in the intercellular space of the papillary cells on the surface and into the stigma (Plate I-4). Then, after 2 h, the pollen tube began to grow down along the intercellular space of the transmitting tissue full of matrices. Eight hours after pollination, the pollen tube grew over the style and while growing in the style, the pollen tube began to utilize the nutritious substances stored in the pollen itself and after a while, imbibe nutrition from the cells of the transmitting tissue. In the process of pollen tube growth, the internal substances gradually gathered up at the end of the pollen tube while the aging pollen tube began to form a kind of cricoid callose tube wall enation in all direction—embolism.

Eight hours after pollination, the pollen tube entered the ovary and grew down along the transmitting tissue in the pseudoseptum. The pollen tube developing inside the transmitting tissue of the pseudoseptum might protrude from different places of the pseudoseptum and then grow along its surface (Plate I-5). When meeting with the ovule, it grew along the ovule surface via funicles. Fourteen hours after pollination, it reached the ovule via micropyle (Plate I-6). The germs and chromatins in the pollen tubes stayed agglutinated but there were still some less agglutinate sperm chromatins. Ma Fengshan et al. (Ma, 1988) observed that the sperm in the pollen tube was long in form while the observed result in this paper showed that it was an ellipsoid sperm (Plate I-7).

3.2 Intromission of the pollen tube into embryo sac and release of its inclusion

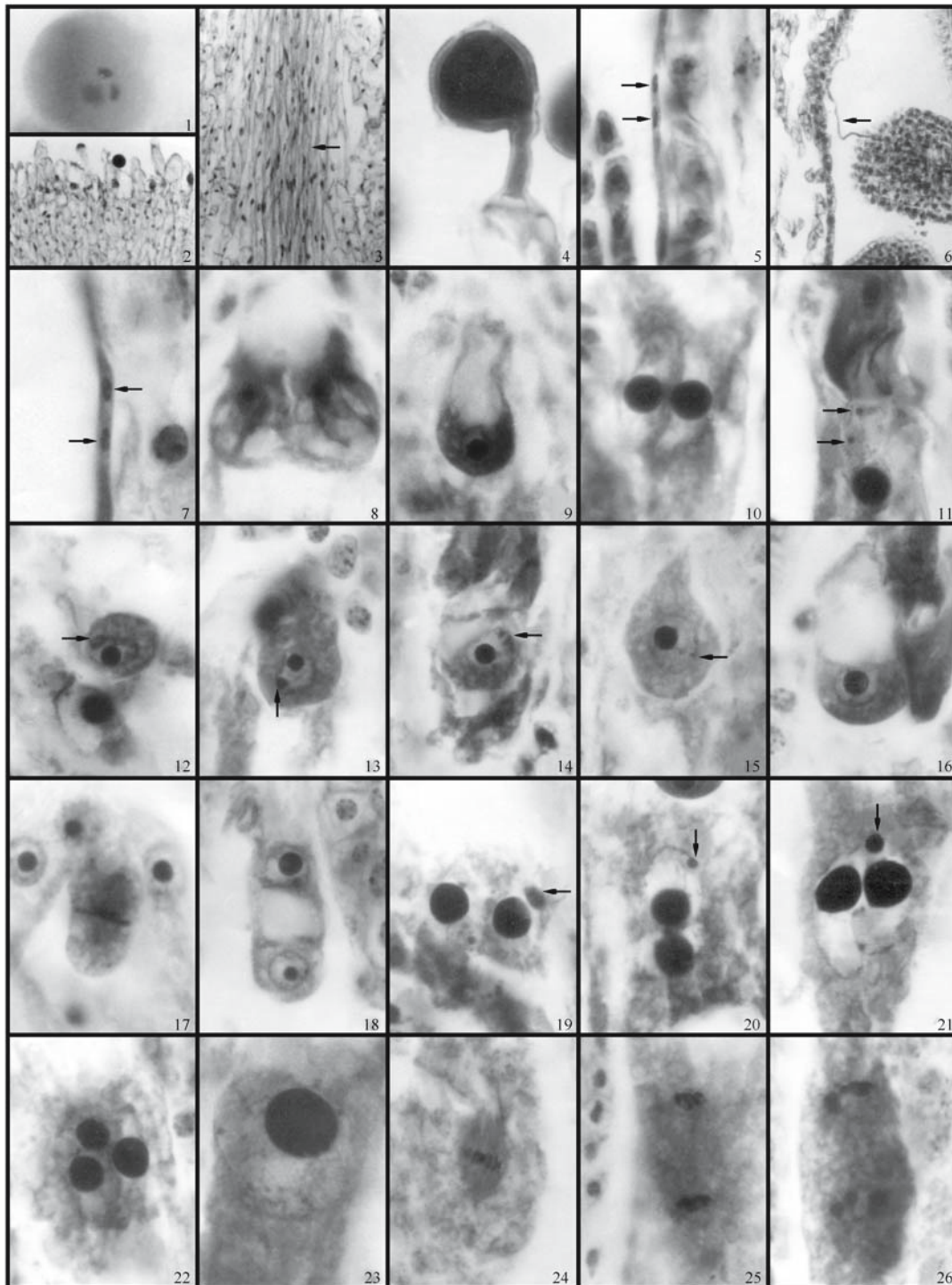
Two synergids in the mature embryo sac adhered to the micropylar end of the embryo sac with darker staining hue and more obvious polarity. Its vacuole and cell nucleus stood

at the chalazal end, and the nucleus stood at the center or next to the micropylar end, respectively. The cytoplasm gathered at the micropylar end. There existed a hook structure outside the synergid and a filiform apparatus at the micropylar end (Plate I-8). The egg lay at the micropylar end of the embryo sac like a Bartlett pear in form (Plate I-9) with distinct polarity and two out of three of the micropylar end occupied by a large vacuole. At the chalazal end stood the cell nucleus and the cytoplasm. The egg nucleus was spherical and the chromatin in the nucleus distributes well with a distinct nucleolus. Two polar nucleuses adhered to each other underneath the egg (Plate I-10). They may be arranged crosswise, horizontally or vertically. Before fertilization, the two polar nucleuses did not fuse or they fused to a secondary nucleus.

3.3 Karyogamy and zygote division

Sixteen hours after pollination, the pollen tube entered the synergid via its filiform apparatus, which was broken and released a pair of sperms. Then, the two sperms moved to the space between the egg and polar nuclei or the secondary nuclei (Plate I-11). After another 2 h, the nuclear membranes of both sperm nuclei and egg nuclei were tightly adhered to each other (Plate I-12), intergradating on the contact surface for another 2 h when the sperm nuclei were transmitted into the egg nuclei. At first the chromatins of the sperm nuclei in the egg nuclei kept their original modality and later in the egg nuclei somewhere, were loosed without the primary ellipsoid (Plate I-13). Twenty-four hours after pollination, the chromatins of the sperm nuclei in the fertilized egg nuclei kept on dispersing (Plate I-14) and the male nucleoli occurred (Plate I-15). As the chromatins of the sperm nuclei disperse, the small male nucleoli were growing larger in volume gradually with full dispersion so that the chromatins of male and egg nucleoli could be hardly separated. At this time, in the fertilized egg nucleolus, a large female nucleolus and a small male nucleolus form a zygote and later, as a result of fusion between female and male nucleoli, a large nucleolus could be seen in most zygote nuclei in the zygote (Plate I-16) but the syncretizing process could not be observed probably because they syncretized so fast. Additionally, some pairs of female and male nucleoli failed to fuse. The loose and disperse of sperm chromatins in the egg nuclei took about 4 h. The karyogamy occurred before the zygote mitosis and the type of fertilization belonged to the premitosis (Hu and Zhu, 1979).

During the zygote dormancy stage, the zygotes elongated gradually to a short rod in form and with the augmentation of the nuclei and their cavity, they further elongated to a long finger in form. The cytoplasm and nucleus began to move towards the chalazal end. 32–34 h after pollination, zygotes began to divide (Plate I-17) into two-celled proembryos (Plate I-18). During the first zygote cleavage, the endosperm was just at the stage of 64 free nuclei. The zygote dormancy stage lasted from 24 h to 32–34h after pollination and lasted for 8–10 h.



1: Three-celled mature pollen; 2: Papillose cells; 3: Transmitting tissue; 4: The pollen tube grows in the intercellular space of papillose cells; 5: Pollen tube grows on the surface of pseudoseptum; 6: Pollen tube protrudes from the pseudoseptum and grows along the surface of pseudoseptum towards ovule; 7: A pair of ellipsoid sperms in pollen tube; 8: Two synergids; 9: Egg; 10: A pair of polar nuclei underneath an egg; 11: A pair of sperms released into the space between the egg and polar nuclei; 12: Lens-shaped sperm nucleus on the surface of the egg nucleus; 13: Karyogamy; 14: Male chromatin disperses in the egg nucleus; 15: Male nucleolus occurs in the egg nucleus; 16: Zygote; 17: Metaphase of zygote mitosis; 18: Two-celled pro-embryo; 19: Two polar nuclei lean each other, sperm nuclei adhere to the nuclear membrane of one of polar nuclei; 20: Male nucleolus occurs in the polar nucleus just now; 21: Male nucleolus increases volume gradually; 22: After triple fusion of sperm and polar nuclei, three nucleoli occur; 23: Primary endosperm nucleus, a big nucleolus in it resulted from the fusion of the three nucleoli; 24: Metaphase of primary endosperm nucleus mitosis; 25: Anaphase of primary endosperm nucleus mitosis; 26: Telophase of primary endosperm nucleus mitosis; 1. $\times 1\ 200$; 2-5. $\times 125$; 6. $\times 800$; 7-26. $\times 1\ 000$

Plate I Fertilization process of *Brassica campestris* ssp. *pekinensis*

3.4 Karyomixis of sperms with two polar nuclei or secondary nuclei and karyokinesis of primary endosperm nuclei

The process of karyomixis of sperms with two polar nuclei or the secondary nuclei seemed as similar as that of female and male gametes in morphology. Although the contact of the former occurred at almost the same time as that of the latter, the former fused more quickly than the latter. Actually, 18h after pollination the sperm nucleus adhered to the nuclear membrane of one polar nucleus or the secondary nucleus (Plate I-19), when the spherical sperm nucleus gradually became lentoid. When it entered the polar nucleus or the secondary nucleus in 20 h after pollination, its chromatin began to loosen and disperse and the male nucleolus occurred (Plate I-20) and its volume increased (Plate I-21). Twenty-two hours after pollination, the sperm chromatin could not be distinguished from that of the polar nuclei or the secondary nuclei completely, when the primary endosperm nucleus occurred. As to the primary endosperm nucleus, there were three conditions: one belonged to the primary endosperm nucleus with three nucleoli (Plate I-22) resulting from the contact between the sperm and two polar nuclei; another was the product of the contact between the sperm and the secondary nucleus, with two nucleoli, one of which was a big secondary nucleolus and the other was a small male one; and the last was the one with one macro-nucleolus (Plate I-23), the result from the fusion among all the nucleoli.

Twenty-four hours after pollination, the primary endosperm nuclei began to divide. The pictures photographed during the whole mitosis process from prophase to metaphase (Plate I-24) and to anaphase (Plate I-25) were very clear. In the two newly formed free nuclei of endosperms, three nucleoli (Plate I-26) were also visible, two of which were big and one small. And later the big ones might fuse into a bigger nucleolus while the small one remained the same. Furthermore, both the big and the small might fuse to create the biggest one. At this time, the next mitosis of the two free endosperm nuclei occurred, which was the same as the findings by Shen Jiaheng (Shen, 1983) in the observation of soybeans. Additionally, the mitosis of the primary endosperm nuclei of Chinese cabbages proved to be prior to that of zygotes without exception.

3.5 Fertilization process and duration of each stage

Three varieties of Chinese cabbages were selected for experimentation for three running years, when the average temperature in the florescence was 20°C at night and 30°C in the daytime. On this condition, the relative duration of each stage in the fertilization process of the three varieties remained the same. It indicated that there was no significant difference among the varieties on almost similar temperature conditions during the florescence.

Usually, 20–30 ovules grow in the ovary of Chinese cabbages. The different places of ovules in the ovary decided the sequence of fertilization. As a rule, the ovule next to the

style was fertilized first and others were fertilized in turn downwards so that the durations from the pollination to the fertilization were somewhat different. According to the integrative observation of 50 000 ovules before or after fertilization, an approximate time table of the durations at each stage in the process of Chinese cabbages is listed (Table 1).

Table 1 Duration of each stage of fertilization in *Brassica campestris* ssp. *pekinensis*

Process of fertilization	Duration/h	From pollination to the first division	Time /h
Pollen grain germinates	4	Zygote division	32–34
Pollen tube grows in the style	4	Primary endosperm nucleus division	24
Pollen tube grows in the ovary and enters ovule	6		
Pollen tube releases sperms in synergid	2		
Two sperms move to egg and polar nuclei	2		
Sperm-egg fusion	6		
Triple fusion of sperm and polar nuclei	4		

4 Discussion

The growth of pollen tubes of Chinese cabbages in the stigmas and styles, observed in this research, proved to be similar to what was described by Chen (1984) and Zhang et al. (1991) but that in the ovary was somewhat different from their description. They suggested that the pollen tubes began to disperse on entering the ovaries and grew downwards along the internal walls while this paper suggested that there existed transmitting tissue differentiation in the ovary pseudoseptum, connecting to the transmitting tissues in the styles. Once growing through the styles, the pollen tubes went into the ovary pseudoseptum and grew downwards along the transmitting tissues. During this course, it might disperse from different places of the pseudoseptum and continue to develop on its surface, and then, when met with the ovules, move along their funicles and finally enter the ovules through micropyles.

The achievement suggests that a time reference may be provided for the research of transmitting the exogenous genes into Chinese cabbages through the pollen-tube pathway method, which is viewed as a natural passage formed by plants themselves when they grow through pollen tubes after pollination or a transgene means to take the exogenous DNA into embryo sacs for the purpose of the genetic transformation. Such a technology proves to be one of the simplest approaches with high efficiency to apply the molecular biology technique to the routine breeding. The study on both the fertilization and their intervals may come up with a schedule for the application of the pollen-tube pathway method.

In other words, this transgenetic technique may offer some reference in three aspects: (1) With a longer style structure, most pollen tubes of Chinese cabbages grow through the styles 8 h after pollination, and thereafter, at the base of styles

appears crosscutting, on the transverse section of which exogenous DNA is instilled; (2) With the separation of ovary antrum into two parts by the pseudoseptum inside the ovary, the genetic transformation with the method of micro-injection can be effective on only part of the ovules; (3) Biology of angiosperm fertilization (Hu and Yang, 2002) indicates that the cell walls at the chalazal ends of the zygote prove to be of deletion or discontinuity during the time from sperm-egg fusion till zygote division, which meet the need of transmitting the exogenous DNA into zygotes. Therefore, the best duration for the exogenous DNA transmission into Chinese cabbages is viewed to be the stage of 20–34 h after pollination.

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