Isolation of Two Populations of Sperm Cells from the Pollen Tube of Tobacco

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Abstract: Pollen of Nicotiana tabacum L. is bicellular type, a generative cell and a vegetative cell in a mature pollen grain. To isolate sperm cells pollen tube should be induced in advance. Using an in vivo-in vitro technique, the style was pollinated and kept in vivo growing for 37 h and then cut about 3.5-4.0 cm long to be cultured in a medium containing 15% (W/V) sucrose, 0.01% (W/V) boric acid, 0.01% (W/V) CaCl₂, 0.01% (W/V) KH₂PO₄, at pH 5.0. A numerous pollen tubes grew out of the cut end of the style after being cultured for 3-5 h. When the pollen tubes were transferred to a broken solution just containing 9% mannitol some tubes broke and released the tube content into the broken solution including two sperm cells. Two sperm cells just released from the tube are connected with vegetative nucleus (VN) of pollen tube consisting of a male germ unit (MGU). Two sperm cells of tobacco are dimorphism: one is big and the other small. The small one (Svn) connects with vegetative nucleus and the big one only associates with the small one. Two isomorphic sperm cells might be selective fusion with egg cell and central cell during in vivo fertilization (preferential fertilization). When two associated sperm cells were transferred into a solution containing 0.01% cellulase, 0.008% pectinase and 9% mannitol, the association between two brother sperm cells disappeared, and both cells could be easily separated using a micromanipulator. To probe the mechanism of preferential fertilization two sperm cells from one pollen tube have to be separated each other to find the differences at the molecular level. Two sperm cells were respectively collected into two individual populations, each containing over thousand big sperm cells or small one, using a micromanipulator. The two sperm populations will offer a possibility to find the differences between two sperm cells in genes and proteins by using molecular methods, which will help us to understand the mechanism of preferential fertilization and gametic recognition of higher plants, especially in the species with bicellular pollen.

Key words: sperm cell; sperm dimorphism; preferential fertilization; Nicotiana tabacum

Since the first isolation of sperm cells were reported by Cass in 1973, the isolation of sperm cells has been conducted in about 40 species of flowering plants. Among these species, most are tricellular pollen grains in which two sperm cells have formed and can be directly isolated from pollen grains (Russell, 1991). To isolate sperm cells from bicellular pollen grains, the pollen tubes have to be firstly induced, in which the generative cell divides to form two sperm cells. Many isolations of sperm cells from bicellular pollen grains were conducted with an in vivo-in vitro technique (Shivanna et al., 1988), in which the stigma was pollinated and the pollen tubes grew in vivo for some time, and then the style was excised and immersed into a culture solution. When pollen tubes grew out of the cut end of the style, it could release two sperm cells by bursting the pollen tubes.

The isolated sperm cell can be used in in vitro fertilization to understand the mechanisms of fertilization, zygote activation and embryo development (Kranz and Lörz, 1993). Sperm cells for in vitro fertilization need to be viable and high-quality but small number in quantity even just one. The isolated sperm cells can also be used in molecular biology researches to find the differences between two brother sperm cells at molecular level and probe the mechanism of gametic recognition. For this purpose, the number of isolated sperm cells not only requires over thousand to make a cDNA library of sperm cells or to detect surface proteins on the cells, but also requires two individual populations of two brother sperm cells to identify the differences between the both from one pollen tube. In most of the former reports two brother sperm cells mixed in one population. Using these sperm cells, some special genes and proteins of Brassica, Lilium and maize have been isolated and researched (Southworth and Kwiatkowski, 1996; Xu and Tsao, 1997; Xu et al., 1999; Zhang et al., 1999; Singh et al., 2002; Xu et al., 2002). In this paper, we report a protocol for the individual collection of two populations of sperm cells from pollen tubes of bicellular pollen of tobacco, which are especially adapted to intensity molecular research.
1 Materials and Methods

Plants of Nicotiana tabacum L. were grown in a controlled growth room at 20 °C in the dark and at 27 °C in the light for 16 h daylength at Xiamen University. Tobacco pollen is of bicellular type, which contains a generative cell and a vegetative cell during flower anthesis. Although pollen grains could germinate and pollen tubes grow very long when pollen grains were cultured in a medium, the generative cell did not divide to form two sperm cells unless using some special treatment (Read et al., 1993). The generative cell division and two sperm cell formation only occur in a pollen tube growing inside style about 6-8 h after pollination (Tian and Russell, 1997a).

Flowers were emasculated before anthesis and artificially pollinated at anthesis. The style of tobacco is about 3.5-4.0 cm in length and it will take 40-44 h for the pollen tube to reach ovary (Tian and Russell, 1998). The whole style was cut at the 38th hour after pollination and cultured in a modified medium containing 15% (W/V) sucrose, 0.01% (W/V) boric acid, 0.01% (W/V) CaCl₂, 0.01% (W/V) KH₂PO₄, at pH 5.0. The osmolality of the medium was 455 mOsmol/kg H₂O. About just 0.5 cm style was immersed into the medium according to natural polarity. After incubated in the medium about 3 h, a lot of pollen tubes grew out of the cut end of the style. To release sperm cells, the cut end of the style with many pollen tubes was immersed into a bursting solution just containing 9% mannitol, pH 5.0. The osmolality of the medium was 493 mOsmol/kg H₂O. Some pollen tubes burst in the solution by osmotic shock and pairs of sperm cell were released.

Paired sperm cells released from one pollen tube could be operated using a micromanipulator. Two brother sperm cells of tobacco are different in size, and the small one connects with the vegetative nucleus and the big one is only associated with the small one. The small sperm cell was named Svn and the big one Sua according to the study of Russell’s (1985), in which Svn was connected with the vegetative nucleus and Sua was not associated with the vegetative nucleus. A few of paired sperm cells without conspicuous difference in size were omitted to keep the purity of two groups.

2 Results

The quality of pollen tubes is very important for releasing tube content including two sperm cells. If pollen tubes grew very well, they were uniform in size and apparently vigorous because cytoplasmic streaming with organelles moving quickly. When the styles with the pollen tubes were transferred to a bursting solution, the pollen tubes quickly burst at their tips and the cytoplasm spurted out. In this condition, two sperm cells were easily identified because the tube cytoplasm soon disappeared in the solution and vegetative nucleus (VN) was associated with one sperm cell (Svn). Generally, the small one (Svn) connected with VN was an average diameter of 6.79 µm, the big one (Sua) was just associated with the small one (Sua), was 7.76 µm. If pollen tubes grown out of the cut end of a style appeared badly, the tip of pollen tube swelled, and cytoplasm moving could not be observed. When these pollen tubes were transferred into a bursting solution, only a few of pollen tubes burst, and the cytoplasm was squashed out of the pollen tubes. The two sperm cells could not be identified because the tube cytoplasm surrounded the both. Normally, the pollen tubes growing out of the cut end of style were good quality within in vitro cultured 5 h. If the styles were cultured over 9 h, the pollen tubes grew too long, only a few pollen tubes could burst.

The newly released sperm cells from pollen tubes are generally ellipsoidal and elongated, and then quickly became football-shaped in bursting solution with 9% mannitol. At the beginning, some cytoplasm of pollen tube wrapped two sperm cells and the vegetative nucleus (Fig. 1), which consisted of a male germ unit (MGU) (Fig. 2). Of the two sperm cells, the one initially connected with the vegetative nucleus is almost always the small one. The big one is associated with small one, but is not directly connected with the vegetative nucleus. The vegetative nucleus swelled and broke down quickly. The association of the both sperm cells could keep at least 30 min. There was a spindle-shaped enveloping membrane around the two sperm cells, and the membrane was divided into two rooms in which two sperm cells located individually (Fig. 2). This membrane could keep intact about 20 min with time after isolation, the cytoplasmic material disappeared from membrane which could not be observed clearly under a microscope but still made two sperm cells be associated with each other. When an enzymic solution dripped into broken solution that made broken solution with a final concentration of 0.01% cellulase (Onozuka R-10), and 0.008% pectinase (Serva), the association between the two sperm cells disappeared quickly. Both the cells could be easily separated using micromanipulator (Figs. 3, 4). Generally, it took 30 min to select a hundred of sperm cells from two styles. After the sperm cells were washed twice in bursting solution the organelles of pollen tube around both could be essentially clean out (Figs. 5, 6). Bursting solution containing purified sperm cells was then transferred into an Eppendorf microcentrifuge tube,
which was precooled with liquid nitrogen. Each tube could store sperm cells over thousand. The sperm cells were stored in liquid nitrogen to prepare for the isolation of mRNA or proteins.

The sperm cells also have a developmental change from the beginning of the sperm formation to the end of the journey growing in a style. At early stage, the association between two sperm cells is very weak, and many paired two sperm cells automatically separate in bursting solution in 10 min. During sperm cell maturation, however, the

**Figs. 1-6.** 1. Newly released two sperm cells wrapped by some cytoplasm of pollen tube. Arrow indicates two sperm cells. 2. Twenty minutes later, pollen tube cytoplasm disappears and the arrow indicates an enveloping membrane around two sperm cells. 3, 4. Two sperm cells are being separated using a micromanipulator. The arrow indicates one sperm cell being drawn into flame-drawn capillaries. 5. A population of big sperm cells. 6. A population of small sperm cells. (All of the figures are enlarged 940 times)
association between two sperm cells becomes stronger, and it was difficult to separate two sperm cells even in the solution over 30 min, which reflects a developmental change.

3 Discussion

Among the isolation of sperm cells of higher plants, most are tricellular pollen species in which two sperm cells have formed when flower bloom. The sperm cells of these species could be directly released from pollen grains by osmotic shock or physical grinding, and en masse isolation of sperm cells could be easily reached. The quantity of sperm cells correlated with that of pollen, and more pollen grains release more sperm cells. The sperm cells in these species with bicellular pollen in which pollen grains contain a precursor generative cell and a vegetative cell, are formed in pollen tube, and it is necessary to develop a pollen tube to produce two sperm cells before isolating sperm cells. Therefore, the isolation of sperm cells from bicellular pollen is more difficult than tricellular pollen because of limited pollen tubes growing in a style, and limited pollen tubes burst in assay. By using the in vivo/in vitro method to culture pollen tube containing sperm cells, Tian and Russell (1997a) have isolated sperm cells of tobacco, and in vitro fertilization of tobacco was also tried (Tian and Russell, 1997b). The isolated sperm cells of tobacco were also used in assay of molecular biology screening special genes of male gamete (Xu et al., 2002). Now, two brother sperm cells of tobacco are intently separated and collected into two individual populations containing over thousand purified sperm cells, which will prepare for using molecular methods to find differences between two brother sperm cells on special genes and proteins that may control double fertilization of tobacco.

Since the preferential fertilization of both sperm cells from one pollen tube was found in Plumbago zeylanica in 1985 (Russell, 1985), the phenomenon of sperm dimorphism has been found in many plants (Russell, 1991; Mogenson, 1992; Hu and Tian, 2002). Some pairs of two sperm cells with dimorphism are different in cellular size, and some in organelles content. All of the sperm cells with dimorphism may be the preferential fertilization: one sperm cell specially fuses with egg to produce an embryo and the other fuses with central cell to produce the nutritive endosperm. Unfortunately, so far the preferential fertilization has only been confirmed in P. zeylanica because of the difficulty of techniques. Besides the differences of size and the number of organelles between two brother sperm cells, recently, the difference of surface charge was also found between two sperm cells of P. zeylanica by using a cell electrophoretic method (Zhang and Russell, 1999). Those differences between both sperm cells occurring in other angiosperms may also have an influence on the pattern of double fertilization. The two sperm cells of tobacco have been found dimorphic in pollen tube (Yu and Russell, 1994) and the divergence becomes more evident with development (Tian and Russell, 1998; Tian et al., 2001). The difference may reflect a gametic recognition during fertilization, and needs to be intensively studied with molecular methods.

Preferential fertilization is a very interesting research project in developmental biology of higher plants, but we know nothing about its molecular mechanism. The preferential fertilization of higher plants may involve two possibilities: egg cell and central cell select two sperm cells, or two sperm cells select egg cell and central cell in vivo. For the former, it is necessary to identify the molecular difference between egg cell and central cell, which needs to isolate egg cell and central cell. For the latter, it is necessary to separate two sperm cells from one pollen tube and to find the special genes that control gametic recognition and preferential fertilization of higher plants. Only after individual type of two sperm cells is collected into two separate populations and each over thousand sperm cells, can the difference between two sperm cells be identified by using general molecular methods. Two purified populations of sperm cells can be used to improve the research of sperm cell biology of higher plants from cellular level to molecular level by sensitive techniques such as immunoblotting, elicitation of monoclonal antibodies or the preparation of PCR-amplified cDNA libraries. The utility of these techniques in sperm cell biology of higher plants will enhance our understanding of the unique properties that the male gametes of angiosperms may possess and contribute to an understanding of the nature of preferential fertilization (Zhang et al., 1998). As a precondition of the use of these techniques, the isolation of two individual populations containing over thousand sperm cells is necessary for the research of molecular characteristics between two brother sperms.

References:


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