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Ultramicroscopic Characterization of Mature Pollen Grains of *Habenaria sagittifera*

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Abstract

Transmission electron microscopy was conducted to characterize the mature pollen grains in the massulae of *Habenaria sagittifera* at anthesis to understand its ultramicroscopic features of mature pollen grains of this species. I found that (a) pollen walls on the surface of the massula consisted of several layers, which included the tectum, baculum, nexine-1, nexine-2, and intine, whereas pollen walls within the massula were comprised of less layers, lacking tectum and baculum; (b) both vegetative and generative nuclei in mature pollen grains were predominated by highly condensed chromatin, which occupied over half of the nuclear volume; and (c) the pollen grains did not contain lipid droplets, starch grains, or storage proteins, indicative of the absence of macromolecular storage reserves. In summary, the structural difference between walls on the surface of the massula and walls within the massula, the highly condensed status of the vegetative nucleus, and the absence of macromolecular storage reserves were the most noticeable ultramicroscopic characteristics of mature pollen grains of *H. sagittifera*.

Keywords: *Habenaria sagittifera*; mature pollen grain; pollen wall; chromatin; pollen storage reserve

1. Introduction

Habenaria sagittifera, a member of the orchid family, has several striking macroscopic features, which include small flowers of less than 1 cm in dimension; also the petals of its flowers are arranged in a cross pattern, and pollen grains are connected together to form a type of composite pollen called massula (Chen and Tsi, 1997). However, the ultramicroscopic and/or microscopic properties of the pollen grains that are linked together to form the massula have not been characterized. One of these properties is the adaptation of the pollen walls to the connection of pollen grains within the massula. Pollen walls of free pollen consist of an intine and an exine; the exine comprises a sexine and a nexine; the sexine comprises a tectum and a bacula; and the nexine includes nexine-1 and nexine-2 (Knox, 1984). However, any structural modifications in the pollen wall that are suitable for the occurrence of connection of pollen grains within the massula remain unknown. Another property involves pollen storage reserves. Mature free pollen grains contain starch grains and/or lipid droplets. Entomophilous pollen grains usually store more lipids than starch as flowers open, whereas anemophilous pollen grains tend to accumulate more starch grains than lipid droplets (Hu, 1982). In *H. sagittifera* the massulae are moved to the stigma surface by insects to complete pollination (Suetsugu and Tanaka, 2014). Considering such ecological significance, it is of interest to researchers to know what type of storage reserves the mature pollen grains within the massula accumulate. The third property involves the development level of mature pollen grains within the massula at anthesis. In some plants, two-celled pollen grains are shed, i.e., pollen grains contain a generative cell and a vegetative cell at anthesis. Other plants shed three-celled pollen grains that contain a vegetative cell and two sperm cells. Pollen grains of Orchidaceae plants embryologically examined thus far are of two-cell type (Brewbaker, 1967; Wu et al., 2012; Liu, 2015); however, that of the mature pollen grains of *H. sagittifera* have not been established. The fourth property involves the status of the vegetative nucleus. The nucleus of the vegetative cell in mature pollen grains of the free type tends to become lobed, and its chromatin is highly decondensed (McCue et al., 2011). However, the behavior of the vegetative

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nucleus and the status of the chromatin that the nucleus contains have not been characterized. To understand these aspects, pollen grains in the massula of this species were examined using transmission electron microscopy.

2. Materials and methods

Flowers of *H. sagittifera* were collected in Fujian and Jiangxi Provinces, from August to September in 2013 and 2014, and kept in a 2.5% glutaraldehyde solution (in phosphate buffer, pH 6.8).

Anthers were isolated and fixed in the glutaraldehyde solution for 12 h. A secondary fixation was performed using 1% osmium tetroxide for 12 h. Dehydration was conducted using 10% upgraded ethanol series. The dehydrated samples were embedded in Epon-812 resin and sectioned on an ultramicrotome with glass or diamond knives. Semithin and ultrathin sections were 1.5 μm and 60 nm thick, respectively. For microscopic observation of lipid droplets, starch grains, or storage proteins, semithin sections were stained with toluidine blue, Sudan black B, periodic acid Schiff reagent, and Coomassie brilliant blue. Ultrathin sections for transmission electron microscopy were stained with uranyl acetate for 30 min followed by lead citrate solution for 5 min.

3. Results

Light microscopy showed that the shape of the massula was tetrahedral or polygonal, with a side length of approximately 130 μm (Fig. 1). The massula was composed of a large number of tightly packed grains. The grains, by and large, were either triangular or tetrahedral, with side lengths ranging from 12 to 15 μm and less than 25 μm at the longest axis. Pollen grain orientation was closely arranged with almost no gaps. The pollen wall on the massula surface differed from the wall within. The pollen wall on the massula surface had a rough appearance, unlike the wall within the massula, which was smooth. No starch grains, lipid droplets, or storage proteins were detected by means of histochemistry.

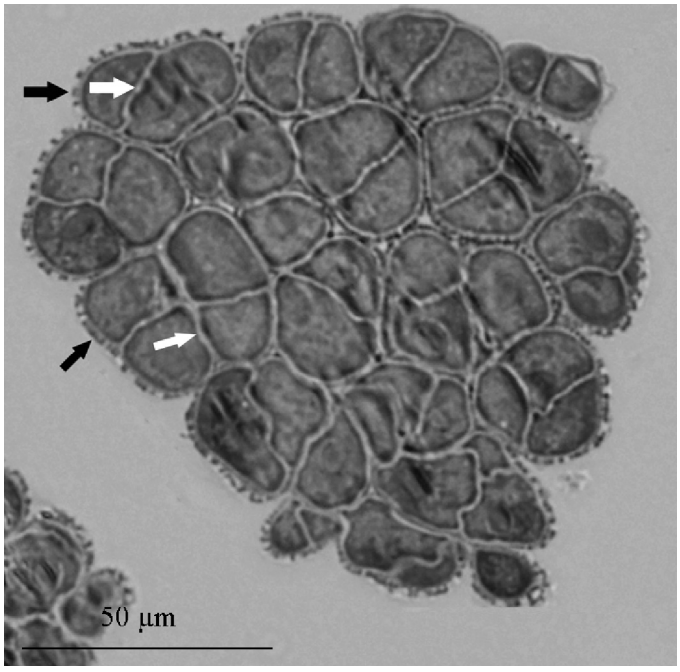

Fig. 1 A massula of *H. sagittifera*. Pollen grains are arranged tightly Pollen walls on the surface of the massula show a rough appearance (black arrows), and pollen walls within the massula have a smooth appearance (white arrows)

Under a transmission electron microscope, the difference between the wall on the massula surface and the wall within was examined in higher detail. The wall on the surface had more sublayers, including tectum, bacula, nexine-1, nexine-2, and intine (Fig. 2, A), relative to that in the wall within the massula, which lacked a tectum and bacula (Fig. 2, B, C). In the wall on the massula surface, the tectum and the bacula comprised sexine, and the nexine-1 and nexine-2 formed nexine. Sexine provided a rough appearance to the wall on the massula surface, as observed under the light microscope. The nexine-1 was less electron-dense than the nexine-2, although the nexine-1 contained rods that were more electron-dense than the matrix (Fig. 2, A). The nexine-2 did not contain such rods and presented an even appearance. The pollen walls of adjacent grains within the massula were fused together (Fig. 2, B). The intine was the innermost layer, which was less electron-dense than the nexine, indicating a loose construction.

The mature pollen grain was of two-celled type, which contained a large vegetative cell and a small generative cell. The vegetative cell took up almost the entire inner space within the grain and the generative cell was enclosed within the cytoplasm of the vegetative cell (Fig. 2, D, E).

The vegetative nucleus showed a smooth surface and was elliptical in shape, with the short axis measuring 4 μm and the long axis 6 μm. The vegetative nucleus was in a highly condensed status: electron-dense heterochromatin occupied more than half the nucleus, whereas less euchromatin was observed (Fig. 2, D, E). Vesicles were the most abundant organelles in the cytoplasm (Fig. 2, C, E). The vesicles were of varied sizes, ranging from 0.1 to 0.5 μm. The vesicles contained a small amount of amorphous inclusions (Fig. 3). There were also endoplasmic reticula in the cytoplasm. Mitochondria had few cristae, and the plastids did not accumulate starch or other types of storage reserves (Fig. 3, A). There were no lipid droplets, which are a type of organelle enclosed by phospholipid monolayer that functions in lipid storage. Ultramicroscopic analysis clearly confirmed that the mature pollen grains did not contain any forms of macromolecular storage reserves.

The generative cell was morphologically highly polarized. It had a broad head and a thin tail that was usually curled (Figs. 2, D and 3,B). Most of the inner space of the head was occupied by the nucleus. The generative cell was 10–12 μm long, the head and tail 5–6 μm long, respectively. The diameter of the head was 4–6 μm, and the diameter of the tail was 1–2 μm. Although the generative cell did not have a cell wall, there was a boundary between the generative and the vegetative cells. The boundary was a thin layer that was basically lucent and contained some amorphous inclusions (Fig. 2, D, E). Similar to the vegetative nucleus, the generative nucleus contained a large amount of electron-dense heterochromatin, but not much euchromatin (Fig. 2, E). The cytoplasm was concentrated. A few small vesicles