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Distribution of starch and neutral lipids in the developing anthers of *Ipomoea cairica*

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The Periodic Acid-Schiff (PAS) reaction was used to test for the presence of starch, and Sudan black was applied to detect neutral lipids (lipid drops) during anther development in *Ipomoea cairica*. Starch was observed in sporogenous and anther wall cells. Prior to meiosis in microspore mother cells (MMCs), starch was not evident, although small lipid drops appeared. A substantial amount of starch accumulated in the epidermal and endothecium cells, but starch and lipid drops were not identified in the tapetum. At the tetrad stage, starch appeared in microspores, and the amount of starch remained constant in anther cells. Early in the microspore stage, the tapetum degenerated, starch remained in the microspores, and lipid drops were completely lost. A large and conspicuous vacuole was formed in the microspores and lipid drops reformed in the cells at the late microspore stage. Following microspore division, the vegetative cell of bicellular pollen grains was filled with starch and lipid drops, and the inner middle layer degenerated. Finally, at anthesis, lipid drops and starch constituted the stored nutritive material in the mature pollen.

Introduction

During anther development, nutritional materials, particularly starch and neutral lipids, are synthesized and transformed in the anthers. Microspore division results in a bicellular pollen grain, and nutritional components begin to be stored in the vegetative cell of the pollen grain before germination. This sequence is closely allied with pollen development, and carbohydrate regulation and metabolism forms the basis of pollen ontogenesis. In some reports, abnormal metabolism of nutritional material in male sterile anthers were demonstrated (Steer 1977, Reznickova & Dickinson 1982, Pacini *et al*. 1985, Ku *et al*. 2003). In fertile anthers of a genic male-sterile Chinese cabbage line, Xie *et al*. (2005) showed starch accumulation in the anther connective tissue, which disappeared during microspore development. However, lipid drops were soon observed in the tapetal cells. During bicellular pollen grain development, lipid

drops aggregated in the tapetal cells and were subsequently detected in bicellular pollen grains. Abnormally large tapetal cells in sterile anthers were also observed and lipid drops did not accumulate during the microspore stage. Later, the tapetal cells stained positive for polysaccharides, but did not concentrate lipids, indicating problems during polysaccharide tapetal cell transformation into lipid drops (Xie *et al*. 2005).

Nevertheless, nutritional material transformation and transportation in anthers remains unclear in higher plants. This is due to reports that the pollen grains of different species store different nutritional components. Therefore, understanding the synthesis and distribution of nutritional constituents in the anthers among different species can provide insights into pollen viability and its role in reproductive fitness.

Ipomoea cairica (Convolvulaceae) is a herbaceous perennial with a nearly worldwide distribution. The species is considered introduced on most continents (its native range is unknown), and is a noxious weed in many parts of the world. However, in Brazil, *I. cairica* has shown efficacy as an antinociceptive (Ferreira *et al*. 2005), and in China it has been used medicinally to treat hypertension and fractures (Jiao & Liu 2009). In this study, *I. cairica* was chosen as a model plant to investigate starch and neutral lipid distribution in developing anthers.

Material and methods

Ipomoea cairica plants were grown in the field on the Xiamen University campus. Anthers were collected at the microspore mother cell (MMC), tetrad, early microspore, late microspore, early bicellular pollen, and late bicellular pollen stages. A minimum of ten anthers from different flowers were collected and fixed, and at least five anthers from each of the above stages were examined. All anthers were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at room temperature; washed in the buffer three times (20 min each); postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2) for 15 h at 4 °C, washed in three changes of the same phosphate buffer (pH 7.2); dehydrated in a graded acetone series; and embedded in an Epon 812 resin. The resin-embedded anthers were cut into 1-μm-thick sections, placed in a drop of water on a clean slide, and mounted on the slide by heating and drying. The methods described by Hu and Xu (1990) were followed to label the sections with 0.5% basic fuchsin (Aldrich, 85734-3) in the Periodic Acid-Schiff (PAS) reaction for 30 min at room temperature. The staining procedure is applied to detect polysaccharides, particularly starch grains, evidenced by pink/red color. The sections were counterstained with 0.3% Sudan Black B (Aldrich, 19966-4) for 30 min at 60 °C to detect neutral lipids (lipid drops), which became black. All sections were observed and photographed using a Leica DMR research microscope.

Results

Sporogenous and microspore mother cell (MMC) stages

During the sporogenous and MMC stages, the anther wall differentiated and was comprised of five cell layers organized centripetally as follows: epidermis, endothecium, two middle layers, and tapetum. The epidermis, endothecium, and middle cell layer exhibited high vacuolization, and one vacuole occupied most of the cell volume. The innermost anther-wall layer was the tapetum, which was in direct contact with sporogenous cells. In the transverse section, the sporogenous cells were arranged in a compact U-shape. Starch grains were located in the sporogenous cells surrounding the nucleus (Fig. 1A). Sporogenous cells underwent transformation into MMCs, with evident increases in the cell size resulting in the development of intercellular spaces between cells. Starch grains decreased during these stages, and lipid material was detected in the cell marginal zone (Fig. 1B).

The most notable feature of the MMCs was the presence of a thick cell wall. This wall exhibited pinkish color, indicating that the *I. cairica* MMCs contain callose and other polysaccharides. The callose (β-1,3-glucan) produced no PAS reaction. PAS is specific to cellulose and starch, among other carbohydrates. The shape of the MMCs was irregular and as the cells

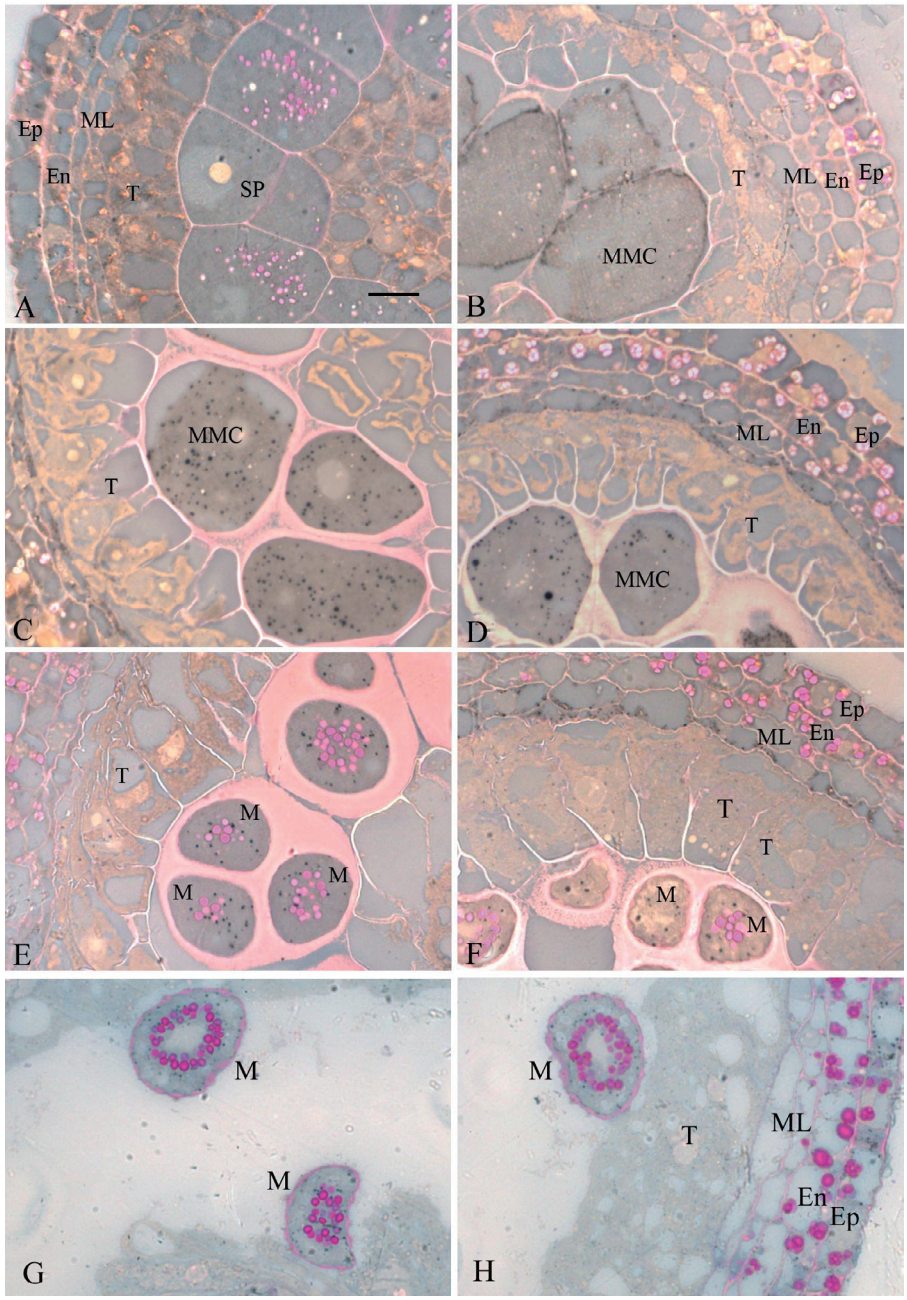

Fig. 1. Starch and lipid distribution in developing anthers of *Ipomoea cairica.* — **A**: Anther at the sporogenous cell stage. Pink spots are starch in the sporogenous cells. — **B**: The absence of starch in early microspore mother cells. — **C**: Microspore mother cells are wrapped in red, lipid drops (stained black) are evident in the MMC cytoplasm. — **D**: MMC stage, pink staining shows starch accumulation in the epidermal, endothecium, and outer middle layer cells, but not in the inner middle layer and tapetal cells. — **E**: Starch, stained pink, is observed in the tetrad microspores. — **F**: At the same stage (tetrad microspores), no evident change in the anther wall cells is evident. — **G**: An early microspore just released from a tetrad; deeply stained red starch surrounds the nucleus. — **H**: At the same stage (early microspore), starch accumulation (stained deep red) remains evident in the epidermal and endothecium cells. The inner and radial walls of the tapetal cells are degenerating. All figures have the same magnification. Bar = 10 μm. Sp = sporogenous cell; Ep = epidermal cells; En = endothecium cells; ML = cells of the two middle layers; MMC = microspore mother cells; M = microspore; T = tapetal cells.

enlarged, the intercellular spaces between them increased in size. This resulted in a complete separation of the MMCs. Starch grains in the MMCs were completely lost and some lipid drops were observed (Fig. 1C). The anther wall contained many starch grains, distributed primarily in the epidermis and endothecium. However, the tapetal cells lacked both starch grains and lipid drops. The cytoplasm exhibited a pinkish color (Fig. 1D), indicating the presence of polysaccharide material.

Microspore tetrad stage

Meiosis in the *I. cairica* MMCs is of the simultaneous type, with four tetrahedral microspores. In the microspore tetrad, starch grains were evident around the nucleus (Fig. 1E). In the anther wall, the epidermis and endothecium cells accumulated starch. In the two middle layer cells, vacuolization increased. Lipid material was observed in the inside middle layer inner tangential wall. The tapetum did not contain starch or lipid materials (Fig. 1F).

Microspore early stage

The four microspores were released when the tetrad callose wall was hydrolyzed. The newly developed microspore was rich in cytoplasm, with a prominent and centrally located nucleus. In this early microspore, lipid drops were present early in development but were lost as development proceeded, and only several starch grains remained around the nucleus. The pollen exine was not formed at this time, but a pinkish polysaccharide material was observed on the microspore surface (Fig. 1G). Starch grains remained aggregated in the anther wall (epidermis and endothecium cells), and very few starch grains were observed in the external middle layer cells. Polysaccharides and starch grains were not detected in the tapetum and internal middle layer. During this stage, the tapetum was characterized by the breakdown of its inner and radial walls, and it subsequently degraded to a protoplastic mass (Fig. 1H). The microspores formed a spinous exine and the microspore cytoplasm contained some starch grains surrounding the nucleus (Fig. 2A). A substantial number of starch grains and some lipid drops remained in the epidermal and endothecium cells of the anther wall. The tapetal cells had degenerated into many small protoplast masses by this point of the early microspore stage. Starch grains were not evident in the middle layer, although lipid drops were observed (Fig. 2B).

Microspore late stage

The formation of a large vacuole characterized the late microspore stage. The nucleus was pushed to the cell periphery, and an evident polarity was formed. In addition to starch grains, lipid drops reappeared in the late microspore cytoplasm (Fig. 2C). The degenerated tapetal cells in the anther wall and lipid drops in the middle layers were not detected. Starch grains decreased in the epidermal and endothecium cells (Fig. 2D).

Early bicellular pollen stage

Unequal microspore division resulted in the formation of a larger vegetative cell and a smaller generative cell. The large vacuole, which occupied most of the vegetative cell volume, decomposed. Large starch grains and small lipid drops were abundant in the vegetative cell cytoplasm of early bicellular pollen (Fig. 2E). Concurrently, the anther wall exhibited no evident change, with a continued accumulation of starch grains and a small number of lipid drops in the epidermal and endothecium cells. However, the inner middle layer cells degenerated, but many large and small lipid drops remained (Fig. 2F).

Late bicellular pollen stage

At maturity, the *I. cairica* pollen was comprised of a vegetative and a generative cells. Therefore, it is classified as bicellular pollen. As the pollen entered the late stages of development, lipid drops in the vegetative cell cytoplasm increased in size and frequency, and were more numer-

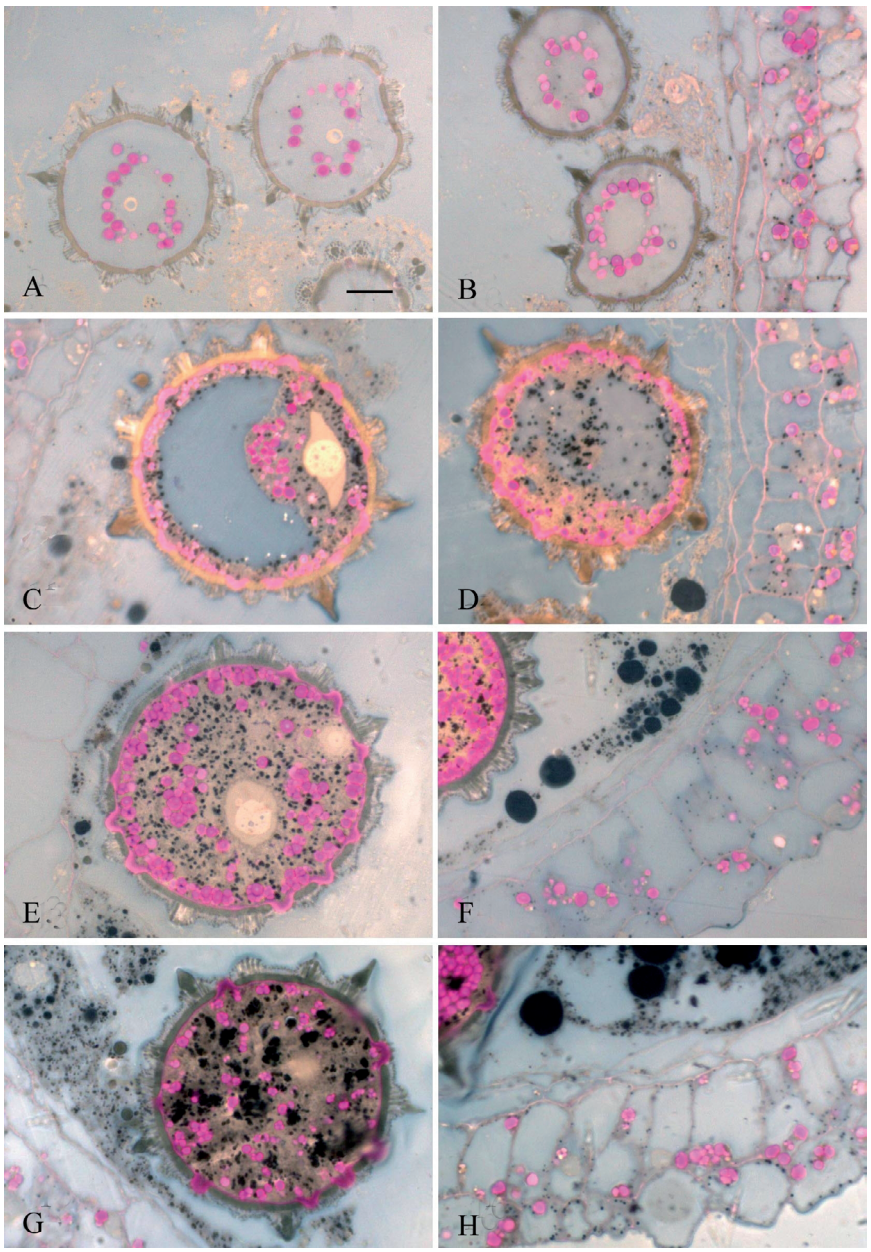

Fig. 2. Starch and lipid distribution in the developing anthers of *Ipomoea cairica*. — **A**: The early microspores show the loss of lipid drops, but the maintenance of starch (stained pink). — **B**: At the early microspore stage, small lipid drops and starch appear in the epidermal and endothecium cells, but starch and lipid drops are absent from the cells of the two middle layers. Tapetal cells have degenerated. — **C**: At the late microspore stage, starch (pink) and lipid drops (black) are present in the microspore cytoplasm with a conspicuous vacuole. — **D**: At the same stage (the late microspore stage), no evident changes are observed in the anther wall compared with the previous stages. — **E**: At the early bicellular pollen stage, after the loss of the large vacuole, the vegetative cell of the pollen grain is conspicuously filled with lipid drops (black) and starch (dark pink), some of which aggregates in the peripheral region. — **F**: At the same stage (early bicellular pollen), the anther wall inner middle layer cells have degenerated and the cytoplasmic remains have transformed into lipid drops (stained black). — **G**: A mature pollen grain filled with lipid drops (black) and starch (dark pink) at nearly anthesis. — **H**: The anther wall consists of epidermal, endothecium, and outer middle layer cells at nearly anthesis. Some starch and small lipid drops remain accumulated in the epidermal cells. All figures have the same magnification. Bar = 10 μm.

ous than starch grains (Fig. 2G). These were the stored nutritional materials for the *I. cairica* pollen. In the anther wall, no evident changes occurred in the epidermis, endothecium, or outer middle layer, and starch grains remained in the former two cell types (Fig. 2H).

Discussion

During anther development, the synthesis and transformation of nutritional components are important metabolic processes. However, the role of these processes remains unclear due to variation in anther development among different species. In the early *I. cairica* anther development, a moderate number of starch grains accumulated in the sporogenous cells. In the MMCs, however, starch grains disappeared and small lipids appeared. Following meiosis, starch was again observed in the cytoplasm and on the cell periphery of the free microspores. Subsequently, the microspore formed a large vacuole, and lipid drops were again detected in the cytoplasm, and on the vacuole surface. Following microspore division, a large number of starch grains and lipid drops filled the vegetative cell of the bicellular pollen. Starch grains and lipid drops exhibiting a routine appearance in developing microspores may serve certain roles. However, the function of neutral lipid material in the MMCs is not clear. Prior to microspore division, accumulated polysaccharides may be related to a specific microspore structure; for example, the formation of a large vacuole may provide osmotic pressure, and be used for pollen intine construction, which is comprised of cellulose. Following microspore division, the accumulated starch grains and lipid drops act solely as carbohydrate storage materials, which prepare the pollen for germination.

The anther wall of *I. cairica* is made up of the epidermis, endothecium, two middle layers, and the tapetum. The tapetum is the innermost layer and attains its maximum development at the tetrad stage of microsporogenesis. All the nutritional materials needed in the locule must pass through the tapetum. It also transforms food materials into forms usable by pollen. During the anther development in *Liriodendron chinense*, the tapetum accumulates substantial amounts of starch, which decrease as starch levels in the microspore increase, indicating polysaccharides are the form of material transformed by the tapetum (Yin & Fan 1998). Xu *et al*. (2006) reported that starch was first detected from the connective tissue in *Lycium barbarum* and served in anther development. Lipid drops subsequently appeared in the tapetum, which suggested the nutritional material transported to the locule was sugar, subsequently transformed into lipid drops by the tapetum, and provided to pollen as a source of nutrients. In the present study, starch and lipid drops were absent from the tapetum of *I. cairica* during the anther development. However, starch grains were consistently observed in the epidermal and endothecium cells, suggesting that tapetal cells rapidly transported sugar into the locule for microspore absorption.

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