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FORMATION OF POLLEN IN THE ANTHER OF LILIUM
II. THE FUNCTION OF THE SURROUNDING TISSUES IN THE FORMATION OF POLLEN AND POLLEN WALL

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SUMMARY

In the *Lilium* anther a peritapetal and tapetal membrane are made by the tapetal cells. During the young microspore stage the pro-orbicules receive a matrix of mainly carbohydrates similar to that of the sexine. The plasma-membrane of the tapetal cells forms membrane-like lamellae. During the vacuolization of the microspore the orbicule diameter increases. In the channels made by the endoplasmic reticulum the globules of sporopollenin are produced. The lipid inclusions accumulate and globules containing carotenoid increase in the plastids. Both elements fuse and form the "Pollenkitt" during degeneration of the tapetal cell. Some sporopollenin globules fuse and form a second generation of orbicules. The cell of the middle layer next to the tapetal cell contains lipid granules. The other four cells of the middle layer lose their starch at the stage of the young microspore and after mitosis the lipid granules are produced. As a source for nutrients in the loculus the starch of the middle layer cells has an important function. An interaction model is schematically presented.

1. INTRODUCTION

In the previous article (part I) the pollen wall formation is described as a continuous process till about four days before anthesis.

During this process four periods of sporopollenin deposition can be distinguished. Exine formation is connected with the presence of membrane-like lamellae, globules and the "Pollenkitt", The cell of the pollen receives its storage products mainly after mitosis of the microspore. These results are related to functions of the surrounding tissues such as source for nutrients and producer of elements for building structures containing sporopollenin. The sporogenous tissue is surrounded by tapetal cells, some layers of middle layer cells, the endothecial cells and the epidermis. Between the tapetal cells and the middle layer cells a peritapetal membrane is present in some plants such as *Pinus* (DICKINSON 1970; WILLEMSE 1971). On both sides of the tapetal cells a wall was observed in some species of *Poaceae* and *Solanaceae* (BANERJEE 1967; AHOKAS 1975: OGORODNIKOVA 1976). The opinion exists that plants with an amoeboid tapetum have a wall between tapetum and middle layer, and plants with a secretory tapetum have a wall between tapetal cell and sporogenous tissue (GUPTA & NANDA 1972). Orbicules are present in many species and are formed by the tapetal cells. The wall between the sporogenous tissue and tapetal cell

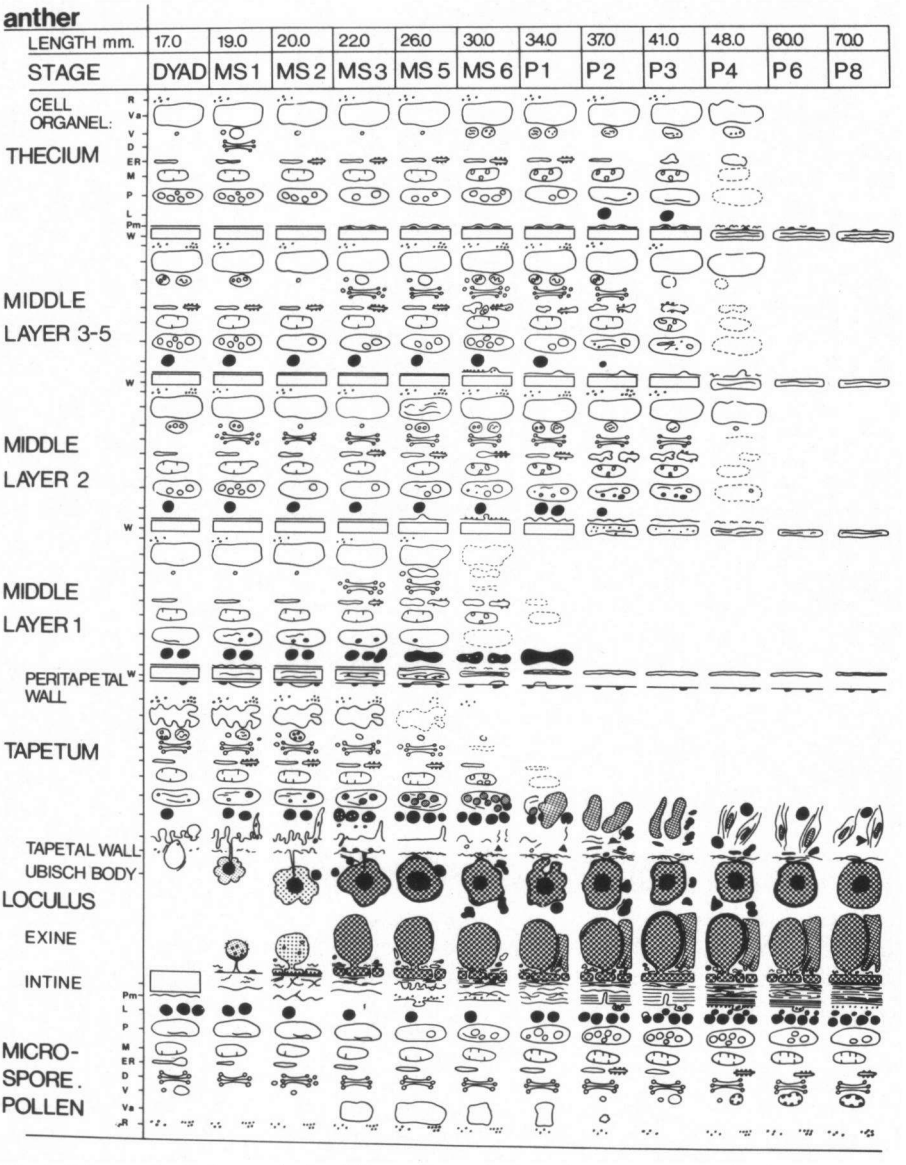

Fig. 1. Scheme of cell changes in the different tissues during microsporogenesis of *Lilium*.
Abscissa: MS1 = microspore just after the release of the callose wall, MS2 = microspore with noticeable wall, MS3 = microspore aquires vacuoles, MS4 = vacuolated microspore, MS5 = late vacuolated microspore, MS6 = late interphase microspore, P_1 = pollen just after mitosis, P2 = young pollen grain, P3 = pollen grain with the generative cell near its wall, P4 = pollen grain with lens-like generative cell, P6 = pollen 4 days before anther dehiscence, P7 = 2 days before anther dehiscence, P8 = 1 day before anther dehiscence.
Used abbreviations: PM = plasma-membrane, L = lipid granule, P = plastid, M = mitochondria, ER = endoplasmatic reticulum, D = dictyosome, V = vesicle, Va = vacuole, R = ribosome and polysome.

should be involved in the attachment of the orbicules (ROMANOV & GRABORSKAJA 1973). About the function of the orbicules different opinions exist (HESLOP-HARRISON 1969; DICKINSON & BELL 1972). MILJAEVA (1966) and BANERJEE & BARGHOORN (1971) suggested that the orbicules serve as a temporary stock of sporopollenin. The tapetal cells, which function in the formation of orbicules, are considered to be a source for sporopollenin precursors (HESLOP-HARRISON & DICKINSON 1969). The "Pollenkitt" is also produced by these cells and contains lipids and carotenoid pigments (HESLOP-HARRISON 1968a, b). The plastids of the tapetal cells form the "Pollenkitt" substances (DICKINSON 1973). The middle layer cells are considered as ephemeral; they disintegrate during pollen development (MODILEVSKY 1963). But it was found that middle layer cells are functionally active after tapetum degeneration (FOSSARD 1969; CHEBOTAR 1972; REZNICKOVA 1973). A relation between endothecium cells and middle layer cells, and microspore development has rarely been considered. This study noted the changes in the ultrastructure of the tapetal and peritapetal wall, tapetal cell, middle layer cells and endothecium in relation to nutrition of the developing microspore and formation of its wall. The function of the tapetal cell in formation of the orbicules, sporopollenin globules and "Pollenkitt" is mainly related to sporopollenin production. A model of interaction, based on some physiological data (REZNICKOVA 1975; HERDT et al. 1978), between the developing microspore and its surrounding tissues, illustrate the formation of sporopollenin structures.

2. MATERIALS AND METHODS

Anthers of *Lilium*, hybrid "Enchantment" were prepared for electron microscopy as described in part I (WILLEMSE & REZNICKOVA 1980). Measurements of the orbicules were made from light micrographs of microspore sections of frozen materials. For each stage the diameter of 100 orbicules was used for curves which express the variation in diameter.

3. RESULTS

The survey of *fig. 1* represents the changes of the different cell organelles of the endothecium, middle layer and tapetal cells. The structure of the orbicules as well as that of the tapetal wall and peritapetal wall are presented. The changes in the microspore and of the pollen wall are included.

3.1. Formation of the orbicules

At the dyad stage pro-orbicular bodies with a diameter in the range of 0.30–0.90 μm are very close to the undulating plasma-membrane outside the tapetal cell. In the loculus electron-dense fibrilar material appears against the pro-orbicular bodies (*fig. 2*).

At the stage of the young microspore, MS1, the pro-orbicular body is surrounded by a layer of loose fibrillar electron-dense material and in contact with

the plasma-membrane (*fig. 3*). This material stains positively for polysaccharides and almost negatively for proteins. Between the undulations of the plasma-membrane fibrils consisting of polysaccharides are present (*fig. 4*).

At the stage when the microspore shows a noticeable wall, MS2, the material around the pro-orbicular body is more electron-dense and homogeneous.

At the stage of the vacuolated microspore, MS4, a thick electron-dense layer surrounds the now electron-dense pro-orbicular body. In some cases an electron transparent layer of about 6–7 nm can be observed on the surface of the orbicule. Outside the plasma-membrane numerous globules probably of sporopollenin are present (*fig. 5*).

During the late microspore interphase, MS6, electron-dense ribbons stick to the orbicules (*fig. 6*). Till the end of the development the orbicule undergoes some morphological changes, the pro-orbicular body remains visible and the border of the orbicule is more electron-dense. The orbicules are weakly positive in polysaccharide tests. A number of orbicules has an irregular shape (*fig. 7*).

3.2. Size of the orbicules

The orbicules are in all stages nearly round and the maximum diameter can be taken as an indication of their size. *Fig. 8* represents the variation in the diameter expressed as the percentage of the total number counted for six selected stages and divided over six classes of a distinct diameter. The diameter of the orbicule increases from the stage of the young microspore, MS2, to the stage of the late vacuolated microspore, MS5. From this last stage till the stage of the pollen with

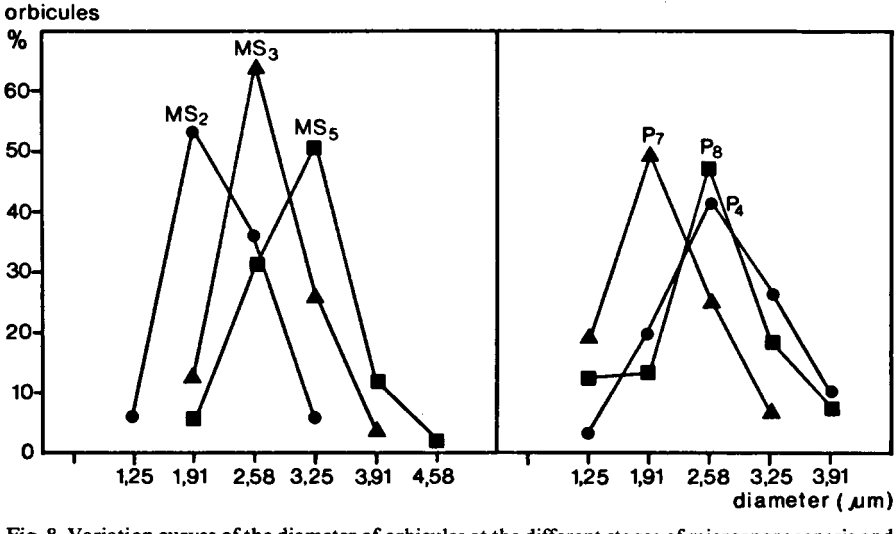

Fig. 8. Variation curves of the diameter of orbicules at the different stages of microsporogenesis and gametogenesis.

a lens-like generative cell, P4, the diameter of the orbicule shows no change in its maximum size and the distribution over the different classes.

At the stage of the pollen with a lens-like generative cell, P4, the maxima of the diameter decreases and the distribution of the values shows a larger variation. A smaller type of orbicule is present as a second generation. One day before anthesis, P8, an increase in the size can be ascertained.

3.3. The tapetal wall

The tapetal cells lose their wall at the tetrad stage. At the stage of the young microspore a layer of fine fibrillar material appears with some electron-dense spots between the tapetal cell and the developing orbicules. From this early moment the orbicules stick to this new and thin tapetal wall (*fig. 9*). In this region material reacts weakly positively to the test for polysaccharides as fibrillar structures (*fig. 4*). When the tapetal cells disintegrate just before microspore mitosis, MS6, their walls consist of a thin layer of a fibrillar substance in which the electron-dense spots are more frequent. At this stage electron-dense ribbons as well as globules, present since the vacuolated stage, are situated on the side of the tapetal cell near the tapetal wall (*fig. 10*). This character of the tapetal wall remains unchanged. On sections of only fixed material the tapetal wall is visible as an electron-transparent line (*fig. 11*).

3.4. Changes in the tapetal cell

At the dyad stage and the tetrad stage the tapetal cells are stretched radially. During the dyad stage the plasma-membrane undulates at the locular side and vesicles are excreted (*fig. 2*). The cytoplasm contains ribosomes, vesicles and some vacuoles. The dictyosomes produce vesicles but the endoplasmic reticulum (ER) is hardly developed. Mitochondria are large and have many cristae. The plastids contain electron-dense globules of about 30 nm. Lipid granules are rarely dispersed in the cytoplasm.

During the young microspore stage, MS1, MS2, the plasma-membrane is undulated and lobed. In the cytoplasm polysomes appear, dictyosomes form two types of vesicles and the ER is present in cisternae of irregular shape. These cisternae form channels in which electron-dense material is present. The mitochondria have pronounced cristae. The dark globules in the plastids reach a diameter of about 80 nm (*figs. 12, 17*).

At the early vacuolated stage, MS3, the tapetal cell becomes more isodiametric and has a large lobed nucleus. The plasma-membrane is less undulating but it forms invaginations when sporopollenin is present on the folds of the plasma-membrane. Such folds can be seen outside the plasma-membrane in contact with electron-dense globules (*fig. 13*). In the plastids the globules, now about 0.7 μm in diameter, are in contact with short tubular structures. A high number of lipid granules (2 μm) appear (*fig. 14*).

At the late vacuolated stage, MS5, MS6, the tapetal cell starts its disintegration. The cell organelles begin to disappear, the contents of the plastids are set free, globules fuse with the lipid granules and form the "Pollenkitt". The con-

tents of the plastids react positively to the test for carotenoids (*fig. 15*).

From the late microspore interphase, MS6, numerous membrane-like lamellae nearly covered with sporopollenin and also ribbons, electron-dense globules as well as "Pollenkitt" are present near the tapetal membrane. Fine tubular elements originating from the plastids are situated between this material (*fig. 16*).

3.5. The peritapetal wall

At the dyad stage electron-dense material is present against the cell wall of the first cell of the middle layer, outside the plasma-membrane of the tapetal cell. Fine fibrillar material is laid down on this electron-dense material to form electron-dense globules (*fig. 17*). This thin peritapetal wall remains present till anthesis.

3.6. Changes in the middle layer and the endothecium

Besides connecting tissue, the anther wall consists of 4 or 5 rows of middle layer cells, the endothecium and epidermis. Except for the layer against the tapetal cells the middle layer cells are connected by plasmodesmata.

The middle layer cells against the tapetal cells, the middle layer 1, can partly be compared with the tapetal cells. During the first stages of microsporogenesis, MS1 – MS4, the vacuolated cell has few ribosomes and ER, the plastids contain some electron-dense granules and the lipid granules accumulate (*fig. 17*). At the stage before mitosis, MS6, more membranes and some dictyosomes develop. In the plastids some large electron-dense globules are present. Many lipid granules originate and these will fuse. Before mitosis the cell disintegrates.

At the dyad stage the cells of middle layer 2 are characterized by the presence of polysomes, polyvesicular bodies, some ER, amyloplasts, few lipid granules and a big vacuole. The amyloplasts lose their starch before vacuolization in the microspore, MS2, but it accumulates again before mitosis. After mitosis of the microspore, the cells of middle layer 2 show vesicles outside the plasma-membrane. The cells contain polysomes, protein containing vesicles and ER. The mitochondrial cristae dilate and the amyloplasts obtain some electron-dense globules. The number of the lipid granules increases (*fig. 18*). When the pollen grains have a generative cell against the wall, P3, the cells of middle layer 2 start to degenerate and lose their starch and lipid granules.

The pattern of cells in the middle layers 3, 4 and 5 are comparable. At the dyad stage they are similar to middle layer 2 cells, but they contain more starch. The loss and restoration of starch occurs at the same moment as in the middle layer 2, at MS2 and at the microspore mitosis.

Before mitosis of the microspore, MS6, some vesicles with protein appear. The ER forms large vesicles and dictyosomes produce vesicles (*fig. 19*). Some accumulation of lipid takes place. After mitosis, P1, multivesicular bodies appear, gradual loss of starch begins and some electron-dense vesicles appear in the plastids. The lipid granules disappear. When the cell starts to degenerate, dilatation of cristae in the mitochondria is one of the first signals for the decay. The cell is disintegrated at the stage that the microspore gets a lens-like generative

cell, P4, following the loss of starch and lipid granules.

Except for the cell volume and the thickness of the cell wall in certain places, the cellular composition of the endothecium cell is comparable with the middle layer cells 4 and 5. Only the changes in the ER and dictyosomes are less clear. The changes in the starch and lipid granules occur during the same period.

4. DISCUSSION AND CONCLUSION

Based on transverse sections of mainly the outer sides of the anther of *Lilium*, the composition of the anther can be described as sporogene tissue surrounded by a tapetal wall, the tapetal cells and a peritapetal wall. These surrounding tissues contain mainly lipid granules as storage product. The next zone consists of the tissue containing starch of the middle layer and the thecium cells. Both layers are involved in the development of pollen.

4.1. Function of the tapetal cell and formation of orbicules

During the dyad stage the peritapetal wall is locally present in its lipid component. At the stage of the young microspore this lipid layer is complete. Globules containing sporopollenin are laid down against this wall when the microspore vacuolates. This composition is in agreement with the structure of the peritapetal wall in *Pinus* (DICKINSON 1970). This wall is a product of the tapetal cell and functions as a barrier between the tapetal cell and the middle layer. But the first middle layer cells show the characteristics of the tapetal cell. The peritapetal wall is therefore not an absolute border.

At the dyad stage the pro-orbicules appear (HESLOP-HARRISON & DICKINSON 1969). The pro-orbicules are in close contact with the plasma-membrane of the tapetal cell. Against the pro-orbicule a fibrillar structure of polysaccharides as well as proteins is present. The matrix increases at the young microspore stage. The matrix of the orbicule is at that moment the same as the exine matrix (WILLEMSE & REZNICKOVA 1980). During the young microspore stage the plasma-membrane undulates. Production of this membrane originates probably from dictyosome vesicles which have a polysaccharide content. This content is excreted after release between the folds. The material can be related to the formation of the matrix around the pro-orbicules as well as around the sexine. Very thin folds or sheets of the plasma-membrane coated with electron-dense material are visible in some places. These sheets have the same structure as membrane-like lamellae. The flat sheets of the plasma-membrane form the membrane-like lamellae and are set free from the plasma-membrane. The way of folding of the plasma-membrane of the microspore gives the same impression. In this material the origin of the membrane-like lamellae is the plasma-membrane and no other structure such as ER. Outside the plasma-membrane a special surface is formed as a glycocalyx (ROWLEY & SKVARLA 1975). Mainly after release from the plasma-membrane, the membrane-like lamellae are places on which sporopollenin is deposited. They are sometimes visible round the or-

bicules as well as round the sexine, and are connected to the globules involved in nexine formation. The lamellae are also connected to exine formation in relation to the "Pollenkitt" (WILLEMSE & REZNICKOVA 1980; WILLEMSE 1971).

During the young microspore stage the tapetal wall starts to form. This tapetal wall functions as a layer on which the orbicules stick. The composition of the layer has not been determined. The products of the anther wall as well as the "Pollenkitt" pass this wall.

During vacuolization of the microspore, M3, the orbicular bodies achieve the same electron-density as the surrounding matrix. The diameter of the orbicules increases strongly. From structural similarity as well as the indication of its increase it can be concluded that at this moment the formation takes place in the same way as in the exine. Probably the same source is used for sporopollenin.

In the plasma of the tapetal cell in the ER cisternae electron-dense globules are developed. This process is observed in the tapetal cells of *Allium* and *Pinus* (RISUENO et al. 1969; WILLEMSE 1971) and in microspores of *Berberis* (GABARA 1974), where the globules are involved in the formation of the exine. They probably stick to the orbicules too. The globules are partly fused after degeneration of the tapetal cell and form structures similar to the orbicules but somewhat smaller. These globules form a second generation as orbicule-like structures. After the vacuolated microspore stage the formation of the "Pollenkitt" starts. Accumulation of granules containing carotenoids and of tubules in the plastids and accumulation of lipid granules initiate this process. Fusion of the granules containing carotenoid and the lipid granules form the "Pollenkitt". This observation is not in agreement with others in which only the contents of the plastids were considered (DICKINSON 1973). The "Pollenkitt" contributes to the formation of the exine (WILLEMSE & REZNICKOVA (1980). However, the "Pollenkitt" does not stick to the orbicules. Ribbons and globules stick to orbicules. Probably the products involved in "Pollenkitt" formation can also contribute to the second generation of orbicule-like bodies, originating from the fusion of globules. This possibility can explain the decrease and increase in the diameter of orbicules during the last periods of development. At this time the orbicule has an electron-dense border which is similar to the exine.

The tapetal cell has the following functions: formation of the peritapetal membrane; formation of the pro-orbicules; its plasma-membrane allows membrane-like lamellae to originate; ER cisternae produce globules; their plastids with the lipid granules make the "Pollenkitt". From the increase in diameter of the orbicule and exine it can be concluded that sporopollenin is deposited until about four days before anthesis on the exine and till the end of the whole pollen development for the orbicules. The same type of development for the exine and orbicules can be ascertained: the formation of the matrix, presence of membrane-like lamellae and sporopollenin impregnation.

4.2. Function of the middle layer cells

The middle layer 1 has some similarity to the tapetal cell and has lipid granules as the main storage product. The plastids also tend to form some electron-dense granules.

The cells of middle layer 2 have starch and lipid granules as storage products. The cells of the other (3–5) middle layers form starch, have fewer lipid granules and less protein in vacuoles. Degeneration of the cells of middle layers 2–5 starts during the stage of the presence of the generative cell against the wall, P3. Dilatation of the mitochondrial cristae is one of the first signs. However, the sequence of the accumulation and decrease in storage products is remarkable. These products are involved in the microspore and pollen development, mainly the break-down products of starch.

The opinion that the tapetal cells function as nutrient cells implies the formation of sporopollenin or its precursors as one of its functions (HESLOP-HARRISON 1968a; HESLOP-HARRISON & DICKINSON 1969; ECHLIN & GODWIN 1968; MASCARENAS 1975). The middle layer cells and the endothecium are never related to these functions. The locular fluid contains carbohydrates (DICKINSON & BELL 1976) and during the young microspore stage the break-down products of the callose are present. The locular fluid is considered as the main component in which all substrates necessary for the microspore and pollen development are present. The tapetal cells function as a source for the enzymes which together with the substrates in the locular fluid and the products in relation to phenylpropanoid metabolism form the pollen wall (HERDT et al. 1978).

Histochemical and physiological studies on the anther of *Lilium candidum* indicate a change in lipids, carbohydrates and starch in the anther and a large reserve of starch in the middle layer cells and the endothecial cells (REZNICKOVA 1975, 1978). Because of the increase and decrease of starch in the middle layer and endothecial cells, the break-down products of starch of these cells are considered as one of the main sources of microspore and pollen development. With the microspore and pollen development, including its wall, as target, a model of this process in relation to the surrounding tissues can be given. In this model the tapetal cell also functions as transfer cell. The middle layer cells have this function too.

An intermediate product in the loculus may be an active acetate as common precursor of lipids, carotenoids, and probably sporopollenin (ATKINSON et al. 1972). The break-down of the starch can be related to the formation of such a product.

4.3. Tissue interaction in the anther during formation of structures containing sporopollenin

From the young microspore stage the starch of the middle layers 2–5 and endothecium cells decreases and is the main source for the locular fluid till just before the first mitosis. During this time in the cells of middle layer 1 and tapetal cells the lipid granules increase, the plastids accumulate droplets containing carotene. In the tapetal cell the polysomes indicate a possible protein synthesis and the membranes start to form the globules.

The pro-orbicules and the exine are formed as structures containing sporopollenin. First a matrix is built, the membrane-like lamellae are formed and the

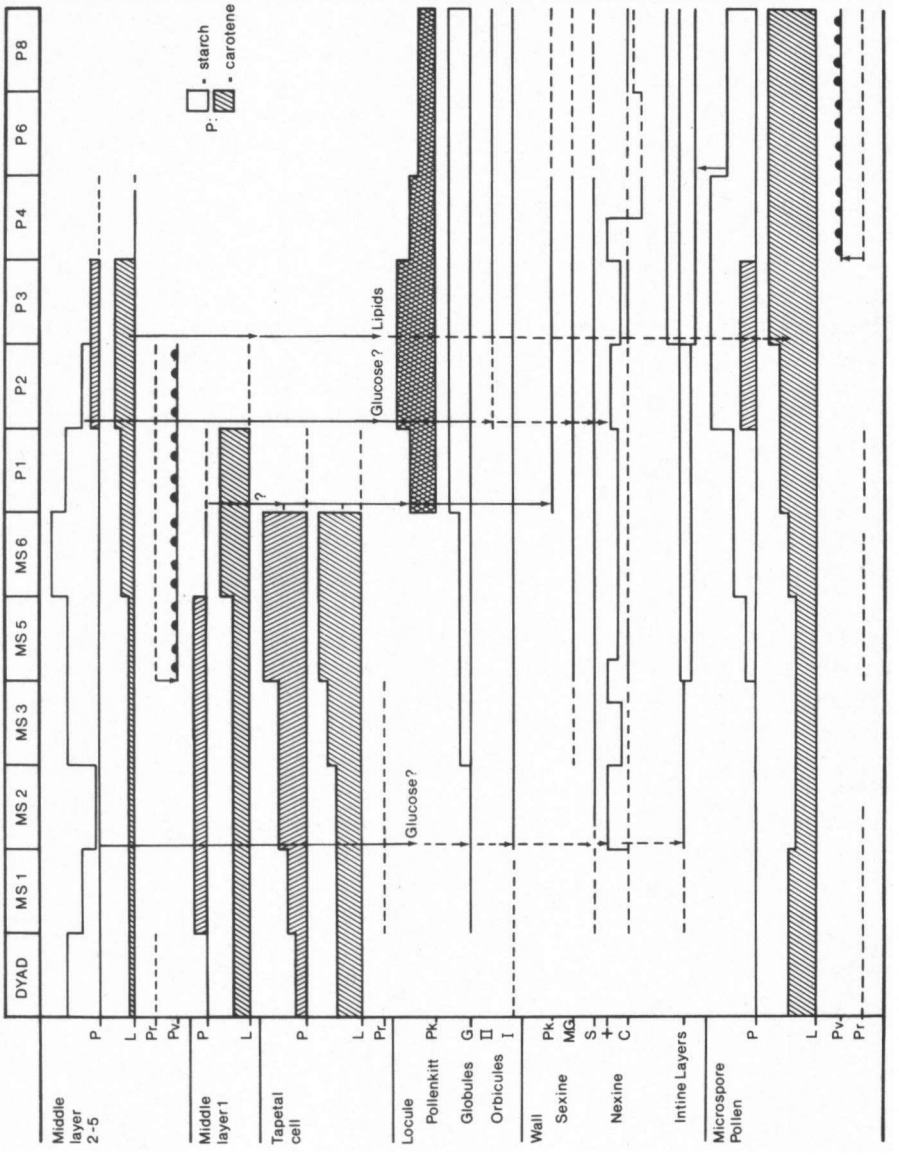

Fig. 20. Scheme of the interrelation between the different developing tissues of the *Lilium* anther. Abbreviations: P: plastids, L: lipid granule, Pr: polysomes, Pv: vesicles with protein. Plc: pollenkitt, G: globules, Orbicules I, II: orbicules of the first and second generation, M: membrane-like lamellae, S: sporopollenin in nexine, + addition to nexine, Nexine c: channels in the nexine.

sporopollenin is embedded in the structures. During this stage the volume of the nexine and orbicules increases twice.

Around the time of mitosis the starch accumulates in the middle layers 3–5 and the endothecium once more. Polysomes are present, polyvesicular bodies and vesicles with protein are formed. Some lipid granules accumulate. The plasmamembrane undulates, an indication that some material is transferred. The tapetal cell and cells of middle layer 1 degenerate and the "Pollenkitt" is formed. The structures containing sporopollenin, the orbicules and exine are in the same state. In the microspore the starch accumulates as well as the lipid granules. After mitosis in the cells of middle layers 2–5 starch decreases, some lipid granules are formed, but the cells start to degenerate. Also the loculus obtains new products such as the lipid granules from the middle layer 1 and the products of starch hydrolysis. The exine is still built up by means of the "Pollenkitt". In the pollen grain the starch decreases, the lipid granules increase and the intine is completed.

Just before anthesis polysomes are present and protein is formed in the microspore. This very simple model is represented in *fig. 20*. Mainly based on ultrastructural changes of the structures containing sporopollenin it indicates a very complicated physiological interrelationship and process.

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LEGENDS TO THE FIGURES

Unless mentioned otherwise the line on the figures represents a length of 0.5 μm.

Plate I.

Fig. 2. Tapetal cell of the dyad stage. Pro-orbicular body close to the plasma-membrane. F: fine fibrillar material, × 45,000.

Fig. 3. Young orbicule with layer of loose fibrillar material. Note the contact with the plasma-membrane, × 27,500.

Fig. 4. Orbicule stained for polysaccharides. The outer layer of the orbicule stains for polysaccharides. Between the undulating plasma-membrane fibrils of polysaccharides are present, × 25,000.

Fig. 5. At the stage of the vacuolated microspore, the orbicule has a homogeneous structure. Note the contact with the plasma-membrane. Outside the tapetal cell membrane-like lamellae (1) and globules are present, × 23,000.

Fig. 6. Late interphase state, ribbons stick to the orbicules (arrow), × 32,500.

Fig. 7. Orbicule towards the end of the development. Near the orbicule many globules (G) and membrane-like lamellae (1), × 18,000.

Plate II.

Fig. 9. Development of the tapetal wall (TW) in the young microspore stage. × 24,000.

Fig. 10. Tapetal wall (TW) just before microspore mitosis, × 49,000.

Fig. 11. Unstained tapetal wall (TW) two days before anthesis, × 10,000.

Fig. 12. Cytoplasm of the tapetal cell during the young microspore stage. Electron-dense material is present in channels of ER cisternae. × 32,000.

Fig. 13. Tapetal cell during vacuolated microspore stage. Sporopollenin on folds of the plasma-membrane (arrow), × 34,500.

Plate III.

Fig. 14. Plastids and lipid granules in the tapetal cell at early vacuolated microspore stage. Note the tubular structures against the carotenoid containing globules, × 22,500.

Fig. 15. Tapetal cell at late vacuolated microspore stage. Pollenkitt (Pk) is formed by fusion of carotenoid globules of the plastids and lipid granules, × 28,000.

Fig. 16. Tapetal cell remnants against the tapetal wall (TW) at the stage just before microspore mitosis, × 18,000.

Fig. 17. Peritapetal wall (PW) with electron-dense globules at the stage of the young microspore and the cytoplasm of the cell of the middle layer 1, × 18,000.

Fig. 18. The cytoplasm of the middle layer 2 at the stage of the young pollen grain with polyvesicular bodies formed in the cisternae of ER. D: dictyosome, × 17,000.

Fig. 19. Cell of the middle layer 4 just before the microspore mitosis. Dictyosome (D), Mb: microbody with protein, × 38,000.

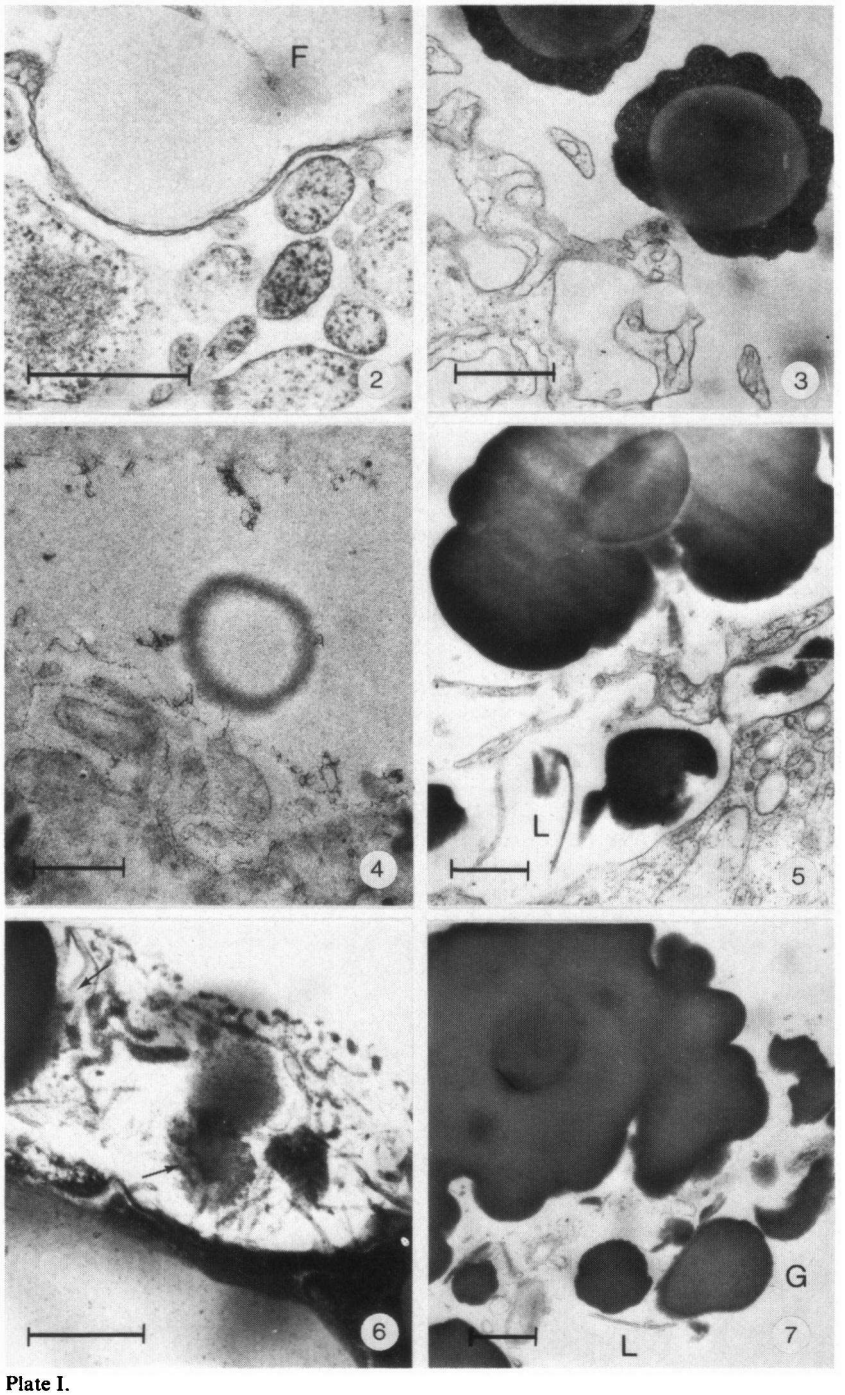

Plate I.

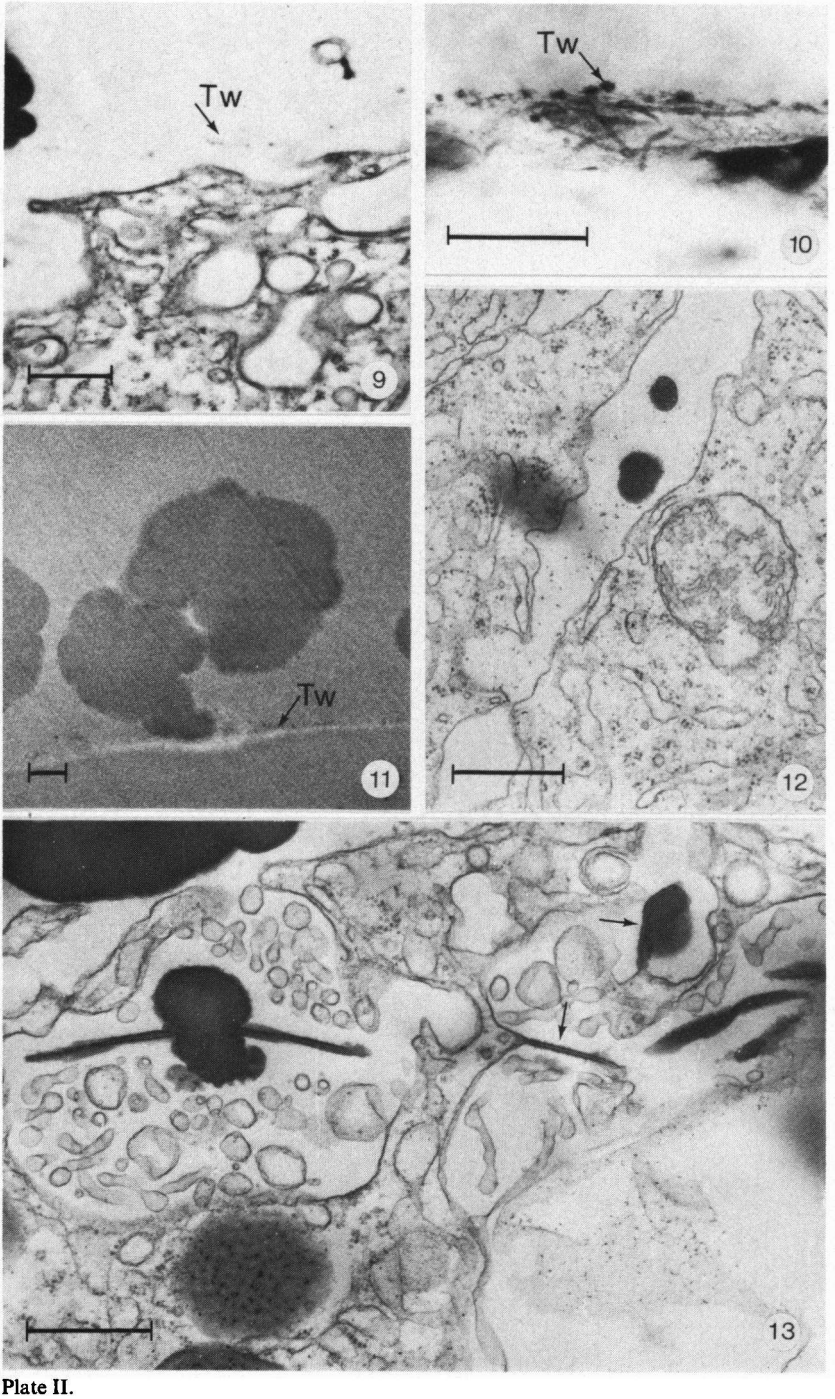

Plate II.

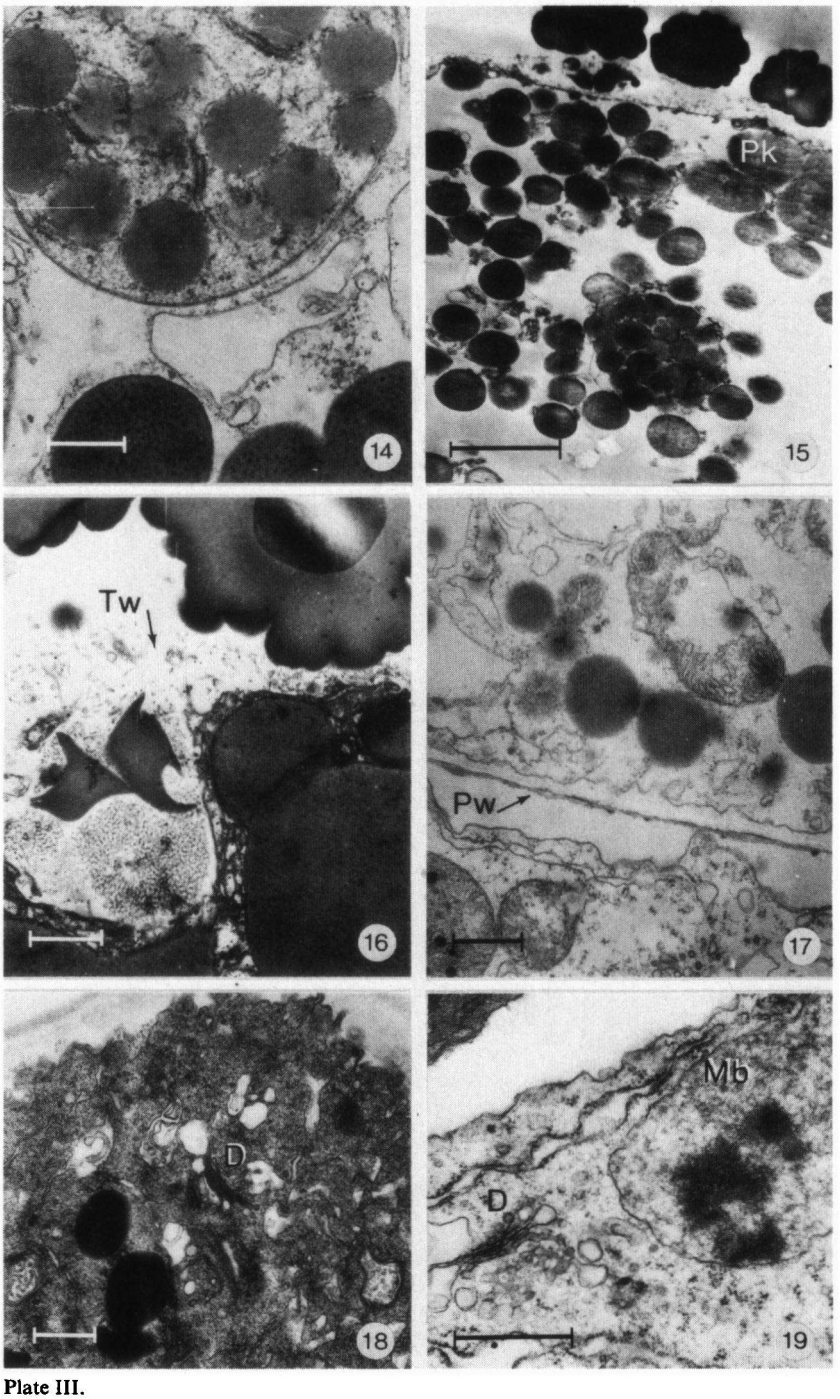

Plate III.