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Callose in the walls of the developing megasporocyte and megaspores in the orchid ovule

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Callose is a polysaccharide discovered in pollen by Mangin (1889) and chemically analysed by Kessler (1958) and defined as β-1-3-*d*--glucane. It has been described in various types of cells where it generally serves as a substance impregnating or bordering the cell walls. It also occurs in the cytoplasm, e.g. in the form of plugs or cocoons in the pollen tube. Most frequently callose has been found in the sieve tubes and at various stages of microsporogenesis (cf. Eschrich 1956; Currier 1957). It is sometimes formed locally in cells impaired or adjacent to lesions, e.g. in carrot callus cultured *in vitro* (Młodzianowski and Szweykowska 1966). A callose layer may appear in no time, the process lasting but a few seconds (Eschrich 1963). It would seem that in many cases callose serves for rapid temporary formation of the cell wall or for its strengthening and after fulfilling this function it may be again resorbed.

In microsporogenesis callose appears in the archespore tissue around the middle meiotic prophase. In this phase the cells of the archespore tissue are connected by numerous thick "plasmodesms", their diameter reaching one micron. The plasmodesms and canals disappear at the end of the first meiotic prophase and the whole wall is callosed. Particularly thick callose walls are observed in the tetrad cells. After the formation of microspores, and as further differentiation of these cells starts, manifested among other things by the growth and formation of vacuoles, callose soon disappears (Eschrich 1962; Waterkeyn 1962, 1964; Heslop-Harrison 1966).

Callose has also been revealed in the megaspore walls of the gymnospermous plant *Encephalartos poggei* of the order *Cycadales* (Waterkeyn 1961; De Sloover 1961).

The here presented results of observations concerning the occurrence of callose in the megasporogenesis of the orchid, starwort and larkspur (the latter two were not analysed in detail) seem to point to a far going similarity, even in the smallest details, as to the time of appearance and disappearance of callose in the processes of mega- and microsporogenesis.

MATERIAL AND METHODS

Ovaries of the flowering orchid *Orchis maculata*, of young starwort buds (*Stellaria graminea* and *Stellaria holostea*) and of the larkspur *Delphinium hybridum* hort. were fixed in ethanol-acetic acid (3:1). Orchid ovlues are very convenient material for following the development of the embryo sac (Strasburger 1923). The ovaries contain an enormous quantity of minute ovules in various development stages. After removal of the ovary walls the rest was hydrolysed in 1 N HCl for 5—10 minutes, then washed with water and squash preparations were made in reagents considered as specific for callose identification. Aqueous resorcin blue solution and 0.05 percent aniline blue solution in aqueous M/15 K_2HPO_4 were used (Eschrich and Currier 1964). Callose stains bright blue in resorcin blue and with anilin blue it gives a yellow fluorescence. For observing the fluorescence a MUF-3M fluorescence microscope was used.

The observations were mainly made on the orchid ovules because it was easier to obtain from them distinct preparations. In the period of megasporogenesis and at the beginning of development of the embryo sac, up to the binucleate stage, these ovules can be readily separated into individual cells. However, the diad and tetrad cells remain coalesced. Metaphase and older megasporocytes and megaspores were the only cells of the orchid, starwort and larkspur ovules which gave an intensive reaction for callose.

It is not excluded that in many cases fluorescence may be utilized for detection of megaspores in intact or squashed ovules.

OBSERVATIONS

Megasporogenesis in *Orchis maculata*.

The megasporocytes differ from other ovule cells by their larger dimensions, dense cytoplasm and large nuclei. In most preparations from younger ovaries, hundreds of cells could be observed in various phases of meiosis. Older ovaries, as already mentioned, were not suitable for making squash preparations.

Preliminary observations showed that the megasporocyte grows intensively in the course of the first meiotic prophase (Fig. 1 *a*, *b*, *c*). Then it undergoes divisions, as a result of which a tetrad of megaspores is formed, its dimensions being about the same as those of the megasporocyte. Neither do the mother cell of the embryo sac and the embryo sac increase in size — at any rate up to the binucleate stage discernable in the squashed preparations — as compared with the chalazal magaspore from which they develop (Fig. 1 *f*—*k*). The cytoplasm of the megasporocyte and of the chalazal megaspore remains dense, however, in the latter

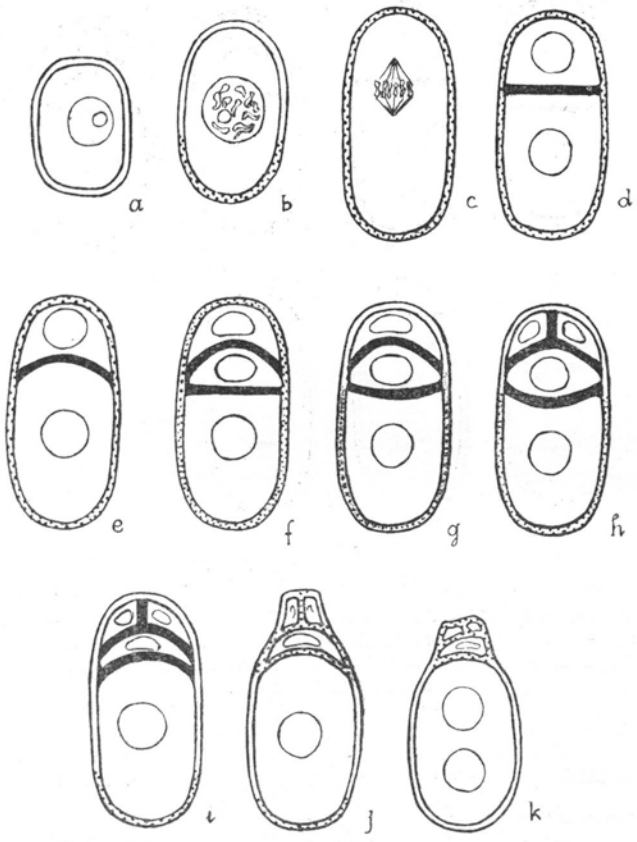

Fig. 1. Diagram of megasporogenesis and incipient megagametophyte development in *Orchis maculata*. After staining with aniline blue some of the cell walls exhibit fluorescence: shaded walls — most intensive fluorescence, dotted walls — weaker fluorescence.

a — megasporocyte in early meiotic prophase; *b* — diakinesis, the chalazal part of the wall fluoresces; *c* — metaphase I; *d* — diad; *e* — older diad with bent transversal wall; *f* — triad composed of two chalazal megaspores and a diad cell retarded in development; *g* — older triad, transversal wall between megaspores bent; *h* — tetrad; *i* — older tetrad upper megaspores begin to degenerate; *j* — further degeneration of megaspores, differentiation of mother cell of embryo sac; *k* — binucleate embryo sac. The proportions of the cells have been approximately preserved in the diagrams

vacuolization occurs, and a large vacuole is already visible between the nuclei of the binucleate embryo sac.

In the first meiotic division two unequal cells constituting the diad are formed (Figs 1 *d*, *e*, 3). The chalazal cell of the diad is generally two times as large as the micropylar cell. Less frequently the division occurs in the equatorial plane of the megasporocyte (Fig. 2 *a*). The second meiotic division occurs nearly always asynchronically. First the diad chalazal cell divides into two unequal parts, the chalazal megaspore being larger than the adjacent sister megaspore (Figs. 1 *f*, *g*; 6). In this way a three-cell row is formed consisting of two megaspores and one diad cell retarded in development. These three-cell

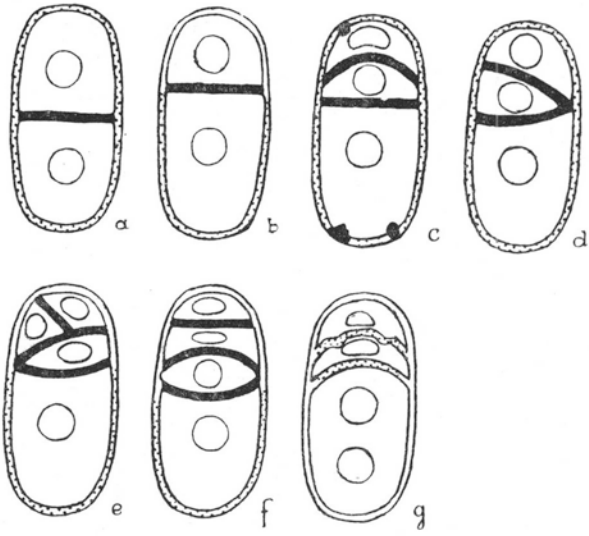

Fig. 2. Diagrams of less frequently observed cell arrangements in megasporogenesis of *Orchis maculata*. Shaded or dotted walls indicate fluorescence.

a — Diad composed of two equal cells; *b* — micropylar diad cell showing no fluorescence; *c* — triad, grains of fluorescent callose in the cells; *d* — slanting postion of separating walls in the triad; *e* — slanting wall between micropylar megaspores; *f* — megaspores arranged in row in tetrad; *g* — binucleate embryo sac with adjacent degenerated megaspore and undivided diad cell.

groups are frequently observed in the preparations. Less often a simultaneous second meiotic division occurs in both the cells of the diad. Even if both the cells divide synchronically, the division of the nucleus in the chalazal cell is nearly always one or two stages ahead of the micropylar cell division. The chalazal cell of the diad divides transversally, and the plane of division of the micropylar cell is usually perpendicular to the plane of division of the chalazal cell. In this way generally a tetrad is formed with cells arranged in T form (Figs. 1 *h, i*; 9). Sometimes only the micropylar cell divides transversally or by means of a slanting wall (Figs. 2 *e, f*). At other times this cell does not divide at all and then the three-cell group may be seen with two degenerating cells and a differentiating chalazal one (Fig. 2 *g*) (cf. S w a m y 1949).

In megasporogenesis, successive changes in the shape of the wall separating the diad cells may be observed as well as between the chalazal megaspore and its neighbour. At first the cells of the diad are separated by a flat wall running perpendicularly to the long axis of the cell. Then this wall bulges out into the micropylar cell (Figs. 1 *d*; *3*) which in cross-section may take the shape of a crescent. The chalazal cell of the diad similarly first divides transversally by a flat wall, which then also bends but this time the bulge is directed towards the chalazal megaspore so that the middle cell of the three-cell group has in cross-section the shape of a lens (Figs. 1 *f, g*). Later, however, some time after

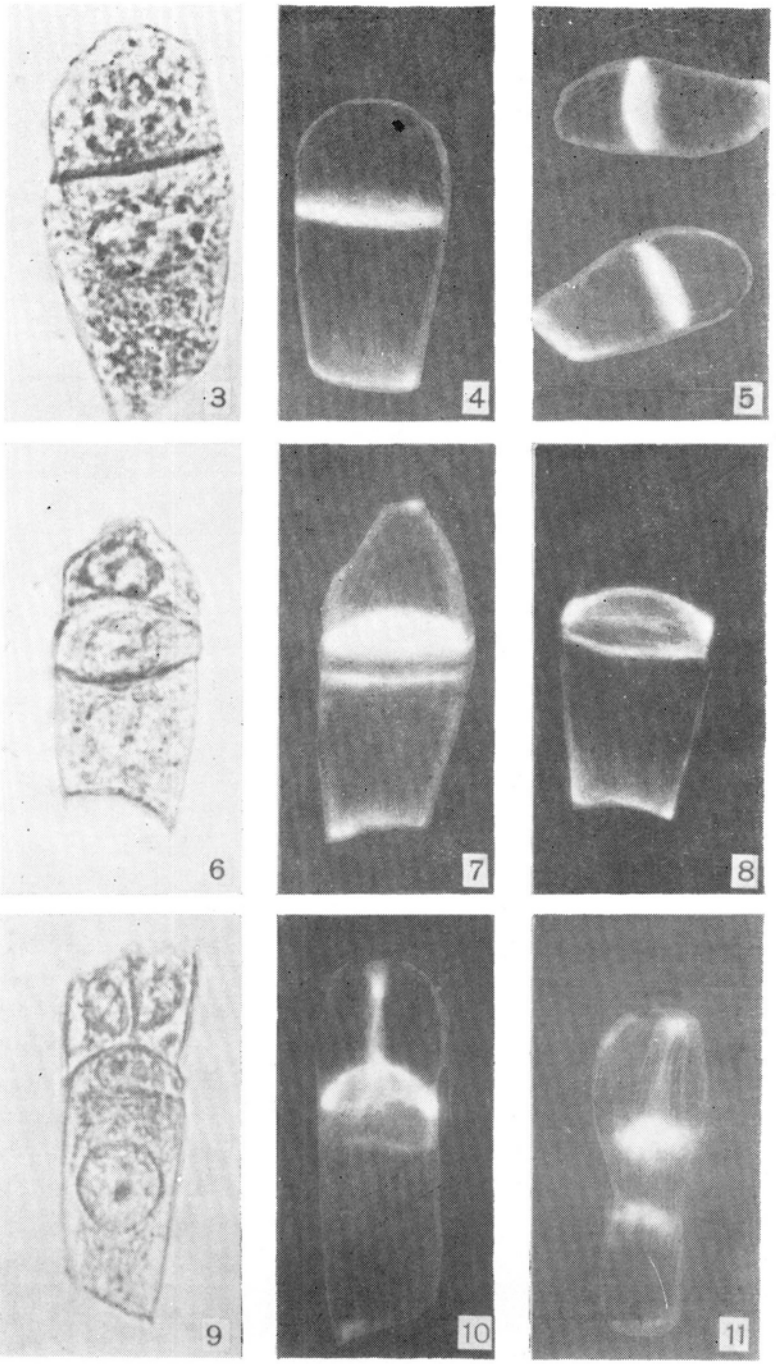

Fig. 3—11. Photographs of *Orchis maculata* cells in various stages of megasporogenesis. Part of the photographs were taken in the fluorescence microscope, fluorescence after staining with aniline blue indicates the presence of callose.

3 — Diad; 4,5 — Fluorescence of cell walls in diad; 6 — Triad; 7,8 — Fluorescence of cell walls in triad; 9 — Tetrad; 10 — Fluorescense of tetrad; 11 — Fluorescence in the ovule of *Stellaria holostea*; tetrad. Magnification approx. 1500, Fig. 5 excepted

formation of the tetrad, the position of the wall changes again and it bulges into the overlying megaspore (Figs. 1 *h*, *i*).

Three of the tetrad megaspores finally completely degenerate, nearly always in the period of differentiation of the embryo sac mother cell, the megaspore adjacent to the chalazal one degenerating first (Figs. 1 *j*, *k*).

CALLOSE IN MEGASPOROGENESIS

Fluorescence signalling the presence of callose appears in the wall of the chalazal pole of the megasporocyte in the midlle period of the first meiotic prophase (Fig. 1 *b*). The fluorescence is rather weak but distinct. Soon, already in the first metaphase, the whole megasporocyte is surrounded by a wall exhibiting distinct fluorescence, generally somewhat stronger on the chalazal side (Fig. 1 *c*).

In the diad stage, the external walls fluoresce more or less similarly as previously in the megasporocyte, however, the newly formed transversal wall separating the diad cells fluoresces much more intensively (Figs. 4, 5). Sometimes the external wall of the micropylar cell of the diad shows weak fluorescence or none at all (Fig. 2 *b*). After the division of the chalazal cell of the diad and formation of the three--cell triad group, strong fluorescence is observable in both the transversal walls but it is much weaker in the external walls which constituted the capsule wall of the megasporocyte. The new transversal wall fluoresces at first weakly and then stronger as the wall bends towards the chalazal megaspore. Then the middle cell is surrounded by a very strongly fluorescing wall (Figs. 7, 8). Observation after staining with resorcin blue seems to indicate that the whole middle cell is lined with a callose layer adhering to the part of the cell wall not containing this substance. In the chalazal megaspore, the chalazal pole generally fluoresces somewhat more intensively as compared to the lateral walls on the whole, however the fluorescence is incomparably weaker than in the middle cell. Sometimes one or more grains of callose material fluorescing in the microscope adher to the wall of the chalazal pole. After staining with resorcin blue, blue grains are visible on the inner side of the megaspore. Similar ones may also be seen in the micropylar cell (Fig. 2 *c*).

After the end of the second meiotic division two megaspores are formed from the micropylar cell of the diad. The wall formed between them exhibits strong fluorescence, whereas the walls adjacent to the somatic cells of the surrounding tissue shine very weakly or not at all (Fig. 1 *h*, 10).

The fluorescence of the chalazal megaspore wall gradually subsides and generally in the early development stage of the embryo sac its entire wall shows no traces of callose. Only at the micropylar pole of

the degenerating or degenerated megaspores a weak fluorescence subsists. In the orchid, owing to the small dimensions of the ovules, fluorescence is well visible in the walls of non isolated megaspores. Similarly the outline of the whole embryo sac may be seen in late phases of development and at maturity, however no fluorescence was noted.

In the larger ovules of the starworts (*Stellaria graminea* and *S. holostea*) and of the larkspur (*Delphinium hybridum*) fluorescence of the megaspore cells was observed. Like in the orchid, these cells were the only structures in the ovules exhibiting strong specific fluorescence. Ovules were noted with one, two, three and four shining cells, mostly arranged in a row. Less frequently the micropylar cell was divided by a slanting or perpendicular wall. In view of the relatively small number of ovules in the ovaries of these plants and the difficulties in their isolation, closer observations were not made.

DISCUSSION

Micro- and megasporogenesis in flowering plants are basically similar processes giving rise, after meiotic division to a spore tetrad. Each microspore then transforms to a male gametophyte, whereas in the monospore type of development of the embryo sac, only one megaspore continues to develop. In microsporogenesis the formation of special walls exhibiting the presence of large quantities of the callose polysaccharide has been frequently observed (cf. Eschrich 1956). This substance appears in the walls of the archespore tissue in the period of the first meiotic prophase and later it appears in the newly formed walls of the diads and tetrads both in the successive and in the simultaneous divisions of the archespore. Successive callose layers surround each microspore and the whole tetrad. It is possible that the callose in the microspores and arechespore cells is synthesized endogenously in the protoplasm. In the megaspores, the callose grains (membrane in the middle megaspore of the triad) and the callose in the chalazal megaspore are also visible on the inner side of the wall. With further differentiation of the microspores the callose gradually disappears.

A similar phenomenon of appearance and disappearance of callose in the cell walls is observed during megasporogenesis in the orchid *Orchis maculata*. Callose forms here in the first meiotic prophase and is most profusely deposited on the walls separating the cells, which arise in meiotic division. It disappears both in the degenerating megaspores and in the chalazal megaspore which is the mother cell of the embryo sac. Beside this essential similarity in the occurrence of callose in micro- and megasporogenesis, certain differences have been noted. The diad and tetrad cells are separated from one another similarly

as in microsporogenesis by strongly fluorescent walls, whereas the outer walls exhibit a much weaker fluorescence or even none at all in the micropylar megaspores. Thus the haploid cells of the generative line are separated from one another by walls much richer in callose than those separating them from the diploid somatic cells.

In the archespore tissue of the anther, the cells in the early meiosis stage are linked by numerous wide canals (W e i l i n g 1965) so that all the protoplasts are bound together closer and more directly than in ordinary meristematic tissue. However, with advancing meiosis, the pits and canals disappear and thick callose walls are formed which isolate all the postmeiotic cells. The isolation of these cells is the more effective, since callose is a highly impermeable substance. It is supposed that the isolation of the postmeiotic cells protects them from noxious interaction which might occur between cells with different genotypes established after the gene segregation (H e s l o p - H a r r i s o n 1966). This argumentation might be applied for interpretation of the role of callose in orchid megasporogenesis, since in the orchid *Dendrobium* disappearance of the pits and plasmodesms in the dividing megasporocyte was observed in the electron microscope, whereas the cells in younger ovules were linked by plasmodesms (I s r a e l and S a g a w a 1965). This would lead to the conclusion that the megasporocyte, the wall of which becomes impregnated with callose and loses its pits as well as the megaspores are isolated from the environment to a high extent.

The chemically modified cell wall probably can not only protect the haploid cells in the case of occurring incompatibility, but by isolating them from the surrounding tissue, eliminates them from the general pattern of regulation. As the result of this isolation the cell follows a different line of development than do the cells of the surrounding tissue. In the ovules of the plants examined, only the megasporocyte and megaspores are surrounded by walls containing callose. This is most pronounced in preparations of whole ovules in which, against the background of nonflurescent tissue, the four megaspores fluoresce brightly.

Callose in the chalazal megaspore disapears in the period of its transformation into the embryo sac, that is at the time when the cell has reached a further degree of differentiation. In the pollen of the *Hyacinthus* of *Chlorophytum* and of other plants there form after the first mitosis two equivalent nuclei, but two different cells differentiate — a generative and a vegetative one. The generative cell is immediately separated after mitosis by a callose wall which disappears within several hours. In the same period the generative cell changes its shape and position shifting from the wall to the centre of the pollen, it seems, therefore, that as the result of isolation, the lines of differentiation of both these cells follow a different course (G ó r s k a - B r y l a s s 1967).

Thus the beginning of differentiation of the megaspores, the embryo sac, the generative cell and the microspores is connected with the periodical occurrence of callose impregnation. The suggestion seems plausible that the concurrence of the beginning of differentiation with the period of chemical changes in the cell walls is not without importance. The changed walls isolate in some extent the cell from other cells of the organism and make possible the development of a new different process. Once the differentiation process has started and is in progress, the isolation disappears.

Another observation requires comment. In the megasporogenesis of the orchid, the walls between the diad cells, and later between the chalazal megaspores change in shape. After both meiotic divisions a wall generally perpendicular to the lateral walls develops (Fig. 1 *d*). Further, when a certain amount of callose has appeared in it, the transverse wall bulges out into one of the sister cells. The direction of this bulge is the same for the pair of cells of the given type (Fig. 1 *e, h*), but it changes in the course of development. These changes may be due to unequal turgor and its changes in the course of development of the diads and of the megaspores neighbouring with each other.

SUMMARY

The presence of callose during megasporogensis of *Orchis maculata* and of three other flowering plant species has been demonstrated by fluorescence microscopy. The fluorescence characteristic of callose is observed earliest in the chalazal wall of the megasporocyte in the period of middle meiotic prophase. Subsequently the whole megasporocyte and later the megaspores are surrounded by callose-containing walls. The disappearance of callose begins in the period of differentiation of the embryo sac from the chalazal megaspore.

It is believed that after the appearance of callose in the megasporocyte and megaspore walls, these cells become isolated in some extent from the surrounding organism. This temporary isolation of the cells may be connected with the beginning of a specific process of development of these cells differing from that in all the other ovule cells. The occurrence of callose in the course of megasporogenesis of the four flowering plant species examined seems to be a process analogous to the formation of callose in microsporogenesis.

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Kaloza w ścianach rozwijającego się megasporocytu i megaspor w zalążkach storczyka

Streszczenie

Metodą fluorescencyjnej mikroskopii wykazano obecność kalozy w megasporogenezie storczyka *Orchis maculatus* i trzech innych gatunków roślin kwiatowych. Najwcześniej obserwuje się fluorescencję charakterystyczną dla kalozy w chala-

zalnej ścianie megasporocytu, który znajduje się w okresie środkowej profazy mejotycznej. Następnie cały megasporocyt, a po tym megaspory są otoczane ścianami z kalozą. Zanik kalozy zaczyna się w okresie różnicowania się woreczka zalążkowego z chalazalnej megaspory.

Przypuszcza się, że po pojawieniu się kalozy w ścianach megasporocytu i ścianach megaspor komórki te w jakimś stopniu stają się odizolowane od reszty organizmu. Okresowa izolacja komórek może się wiązać z podjęciem przez nie specyficznego procesu rozwoju, odmiennego niż we wszystkich innych komórkach zalążka. Występowanie kalozy w procesie megasporogenezy czterech badanych gatunków roślin kwiatowych wydaje się być analogicznym procesem do tworzenia się kalozy w mikrosporogenezie.