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Changes of RNA synthesis in Anther Wall of *Brassica napus* during Male Gametogenesis

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The distribution of RNA in the anther wall of *Brassica napus* during male gametogenesis was followed by ^{3}H-uridine autoradiography. Silver grain (SG) density was not above background in the anther wall just after microspore was released from tetrad callose wall. Significant accumulation of SGs occurred in tapetum, endothecium, and epidermis before microspore vacuolation. Accumulation of RNA in the tapetal cells was peak before the vacuolation occurred in the microspore. With the onset of tapetal senescence at the partially vacuolated microspore stage, SGs decreased and they completely disappeared in the tapetum at the bicelled pollen stage. Accumulation of RNA in the endothecial cells was peak after the microspore mitosis and continued just after the generative cell mitosis. Appreciable SGs also occurred in cells of epidermis from nonvacuolate microspore stage to bicelled pollen stage. During this period, SG density was almost same and was not high as compared with tapetum or endothecium. At tricelled mature pollen stage, no incorporation occurred in anther wall. SGs were found mostly over the nucleolus and chromatin of the cell nuclei.

Keywords: autoradiography, *Brassica napus*, male gametogenesis, RNA synthesis, anther wall

In most angiosperms the anther wall consists of the epidermis, endothecium, middle layer(s), and tapetum. Tapetum, the innermost layer of anther wall, completely surrounds the sporogenous tissue and is of considerable physiological importance because all the food material to the sporogenous tissue must pass through it. It is generally assumed that the tapetum was involved in pollen development (Echlin, 1971). During the early stages of anther development, tapetal cells are characterized by densely staining cytoplasm and prominent nuclei, while at later stages they become enlarged and multinucleate and polyploidy. Eventually, the walls of the tapetal cells break down, followed by migration of the contents into the anther loculus (Bhandari, 1984). The middle layers are generally ephemeral and become flattened and crushed by meiosis in microspore mother cell. The endothecial layer attains its maximum development when the anther is ready to dehisce for the discharge of pollens. During the maturation of anther, the endothecial cells acquire thickenings and the fibrous bands arise chiefly along the inner tangential walls (Bhojwani and Bhatnagar, 1979; Bhandari, 1984).

Besides their traditional role in the production of male gametophyte, anthers are important in the regeneration of microspore embryo by anther or microspore culture (Aruga and Nakajima, 1985; Raghavan, 1986, for review). Especially the presence of the anthers in the initial stages appears to be important (Tyagi *et al.*, 1979; Kasha *et al.*, 1990). Some workers (Kohler and Wenzel, 1985; Ziauddin *et al.*, 1990) concluded that some factor(s) coming from anther walls might induce or stimulate microspore embryogenesis in the initial period of anther culture. Thus, the anther provides an interesting system for examining the control of gene expression during cell specialization in the wall layers.

Recently the advent of molecular genetic techniques has provided the incentive for much work on the cloning and identification of genes that are exp-

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ressed during the development of the anther. Several research groups have been studying gene expression in anther development (Mascarenhas, 1988, 1990), and tapetum-specific genes have been described in tobacco (Goldberg, 1988; Koltunow *et al.*, 1990), tomato (Smith *et al.*, 1990), and *Brassica* (Scott *et al.*, 1991; Theerakulpisut *et al.*, 1991; Shen and Hsu, 1992). More recently, it has been demonstrated that male sterility can be induced in tobacco (Koltunow *et al.*, 1990; Mariani *et al.*, 1990) and *Brassica* (Mariani *et al.*, 1990) using chimeric gene containing a tobacco tapetal cell transcriptional control sequence (TA 29) in conjunction with various cell disruption genes. Nonetheless, biochemical analysis of the anther wall is limited by the heterogeneous nature of its constituent cells and by the difficulty of isolating them. For some purposes, the use of cytological methods for the detection of macromolecules in squashed or sectioned preparations offers a way to overcome these difficulties. This technique has become a convenient tool to identify specific tissues and cells that harbor specific messages in multicellular organs.

A few words about terminology are appropriate here. Although the terms "microspore" and "pollen grain" have been used interchangeably and synonymously in the past, I will use the term microspore in this paper to refer to the product of meiosis of the microsporocyte. The microspores released from the callose wall of the tetrad later divide mitotically (microspore mitosis) to form pollen grains with a large vegetative cell and a small generative cell, and later the generative cell divides (mitosis of generative cell) to form two sperms.

As part of an effort to study the possible role of anther wall during male gametogenesis and microspore embryogenesis ^{3}H-uridine incorporation pattern in the anther wall of *B. napus* during male gametogenesis has been followed by autoradiography. For orientation, the cytological changes in anther tissue that occur during male gametogenesis have also been investigated.

MATERIALS AND METHODS

Plant material

Plants of *Brassica napus* L. cv Topas were raised from seeds in a growth chamber at 22℃ under a photoperiod of 18 h provided by fluorescent and incandescent lights (12,000-13,000 luces at pot level) and allowed to flower under the same conditions.

Permanent preparation

Anthers containing microspore and pollen at different stages of development were dissected from flower buds and immediately fixed in 70% ethanol-acetic acid (3 : 1) for 24 h. The fixed anthers were dehydrated through 90 and 100% ethanol, *n*-propanol, and *n*-butanol, and embedded in glycol methacrylate (Feder and O'Brien, 1968). Anthers were sectioned longitudinally at 7 μm thickness on a rotary microtome equipped with a steel knife. Sections were attached to slides with drops of water and after draining off water, slides were dried for several days at room temperature. To follow normal development of anther wall, a set of slides was stained in 0.1% toluidine blue for 20 s, rinsed in water, and mounted in Euparal.

Autoradiography

To monitor the incorporation of ^{3}H-uridine into cells of anther wall during male gametogenesis, isotope treatment and autoradiography essentially followed the method of Raghavan (1982), as modified in this laboratory for use with plant materials (Kim, 1993). In the protocol followed here, anthers were dissected from flower buds and immediately immersed in the Murashige and Skoog's basic medium supplemented with 10 μCi/mL ^{3}H-uridine (specific activity 29.7 Ci/mmol; Amersham Corporation), and incorporation of the isotope into cells of anther wall allowed to proceed for 2-4 h. Following this, anthers were washed in unlabeled precursor (10 mg/L uridine), and fixed in acetic-alcohol. The fixed anthers were processed for microtomy as described earlier. For autoradiography, unstained slides were dipped in Kodak NTB_3 liquid emulsion which was diluted with an equal volume of water. After exposure in the dark for 28 days, the slides were developed for 6 min in Kodak D-19 developer at 14℃, briefly rinsed in distilled water, fixed in acetic-alcohol, and washed in running water for at least 30 min. They were stained through the processed emulsion with azure B, dehydrated, and mounted in Euparal.

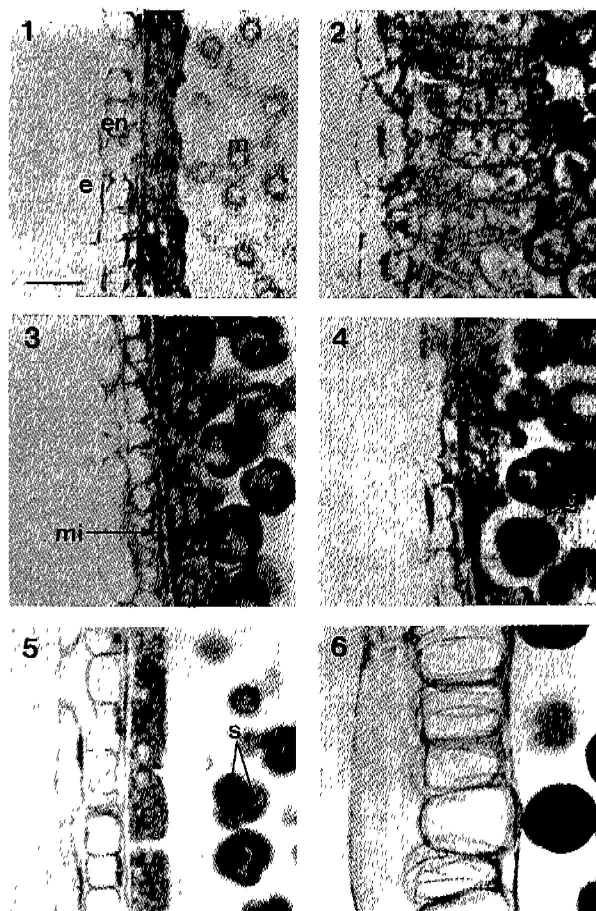
Scoring of slides

For each sampling stage, sections from two or three anthers of the same flower bud were used. Slides were examined on an Olympus BH-2 with X100 oil immersion objective. To determine the extent of ^{3}H-uridine incorporation into cells of the anther wall, the number of silver grains found over the nucleus and surrounding cytoplasm was counted from random samples. Silver grains were also counted from an area adjacent to the labelled cells of anther wall to provide an estimate of the background. For each stage of anther development, counts were made of at least 20 cells from two slides. Sections were photographed on Fuji Microfilm using bright-field optics. The experiments were repeated once with slides made from a fresh sample of anthers, using a new batch of ^{3}H-uridine with the essentially same results.

RESULTS AND DISCUSSION

Development of anther wall during male gametogenesis

During the newly released microspore stage, anther of *B. napus* consists of a well-defined layer of epidermis, followed on the inside by a layer of endothecium, and a layer of tapetal cells (Fig. 1). At this stage, tapetal cells are binucleate and cells of middle layer are already compressed and disorganized and only remnants of middle layer appeared ocassionally. At the time of nonvacuolate microspore stage, tapetal cells are already binucleate and considerable radial enlargement occurred but fusion between daughter nuclei did not occurred (Fig. 2). Multinucleate condition combined with endoreduplication is characteristic of tapetal cells of many plants. At this stage, tapetum attains its maximum development. As vacuolation is initiated in the microspores, tapetal cells separate from one another by breakdown of their radial walls and appear as small elliptical or convolute cells (Fig. 3). During the senescence phase, cytoplasmic contents broke away from the tapetal cells and migrated to the anther loculus. Tapetal cytoplasm and nuclei which invaded the anther loculus rapidly digested, and at the pollen stage, only remnants of the cytoplasm and nuclei attached to



Figs. 1-6. Anther wall at different stages of male gametogenesis. Fig. 1. Newly released microspore stage. Binucleate tapetal cells are seen. Fig. 2. Nonvacuolate microspore stage. Tapetal cells are binucleate and considerable radial enlargement occurred. Fig. 3. Vacuolate microspore stage. Tapetal cells are in the senescence phase. Fig. 4. Bicelled pollen stage. Tapetal cells are almost same as the vacuolate microspore stage. Fig. 5. Immature tricelled pollen stage. Only remnants of tapetal cytoplasm and nuclei attached to anther wall are found. Fig. 6. Mature tricelled pollen stage. Tapetal cells are completely dissapeared. Fibrous bands in the endothecium are developed. In Figs. 1-6: e, epidermis; en, endothecium; mi, middle layer; t, tapetum; m, microspore; g, generative cell; s, sperm. Calibration bar (20 μm) in Figure 1 applies to all the figures from 2 to 6.

the anther wall were found (Figs. 4 and 5). At the mature tricelled pollen stage, tapetal cells disappeared completely but endothecial cells developed extensive thickenings and fibrous bands were developed (Fig. 6). The endothecium along with the epidermis persisted in the mature anther and at mature tricelled pollen stage, anther wall consisted of an epider-

mis and endothecium.

The noteworthy feature of the study of normal anther wall development presented here is that the improved resolution of the cytoplasmic contents of the cell obtained by embedding them in glycol methacrylate has made it possible to provide a description of the cytological changes during anther development which will serve as a framework of reference for following ^{3}H-uridine incorporation at the light microscope level. The basic changes in the anther wall development that occur during male gametogenesis in *B. napus* are similar to those observed in other plants of angiosperms (Biddle, 1979; Bhandari, 1984; Goldberg *et al.*, 1993). In angiosperms the tapetum is of the "secretory" or "plasmodial" types according to the condition of the nucleus and cytoplasm at the time of dissolution (Biddle, 1979). Cells of secretory type retain nuclei and much of the cytoplasm, and cells of amoeboid types may possess only remnants of the degenerate cytoplasm and lack nuclei. The time of dissolution is extremely variable. The walls breakdown during meiosis I in *Avena* (Steer, 1977), at tetrad stage in *Beta* (Hoefert, 1971), *Lens* and *Pisum* (Biddle, 1979), and during microspore development in petunia (Bino, 1985). But generally tapetum attains its maximum development at the tetrad stage. The tapetum of *B. napus* is of the secretory type in its essential characteristics and attains its maximum development at the nonvacuolate microspore stage.

RNA synthesis in cells of anther wall

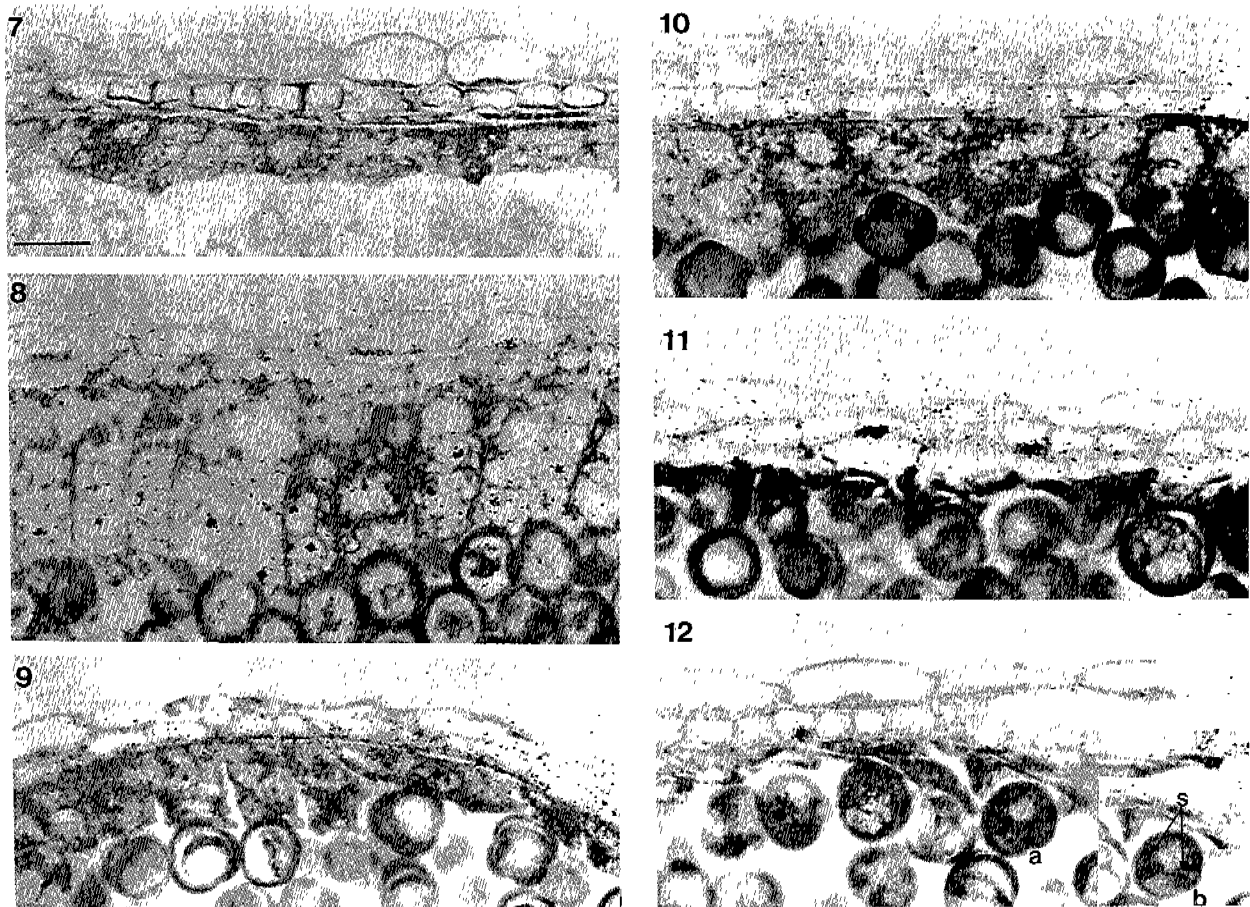
The pattern of distribution of autoradiographic silver grains over cells of the anther wall of different stages following incorporation of ^{3}H-uridine for 2 h is presented in Table 1. The results show that cells of the anther wall at the stage of microspore immediately after release from the tetrad do not incorporate ^{3}H-uridine (Fig. 7). The first detectable incorporation of the isotope is found in cells of the anther wall of the nonvacuolate microspore stage (Fig. 8). At this stage, the density of autoradiographic silver grains due to ^{3}H-uridine incorporation in tapetal cells was peak and significant accumulation of silver grains also occurred in the endothecial and epidermal cells. With the appearance of degeneration of tapetum at partially vacuolate microspore stage,

Table 1. Number of silver grains over cells of anther wall at different stages of male gametogenesis in *B. napus* following incorporation of ^{3}H-uridine

Stages of male gametogenesis	Number of silver grains (± SE) per cell		
	Tapetal cell[a]	Endothecial cell	Epidermal cell
Microspore, soon after release from tetrad	0	0	0
Microspore, before vacuolation	29.4± 3.9	6.1± 1.8	7.8± 1.8
Microspore, during vacuolation	23.8± 3.0	7.3± 1.7	8.7± 2.0
Microspore, after vacuolation	0.3± 0.1	7.1± 1.9	8.4± 1.8
Bicelled pollen	0	22.5± 2.8	7.2± 1.5
Tricelled pollen, soon after generative cell mitosis	0	17.5± 2.2	0
Tricelled, mature pollen	0	0	0

[a]The tapetal cells are binucleate.

there was a noticeable decrease in the incorporation of ^{3}H-uridine in the tapetal cell (Fig. 9). In autoradiographs of anthers sampled at vacuolate microspore stage, silver grain density over tapetal cells was only slightly above the background and at the bicelled pollen stage, these tapetal nuclei did not bind any ^{3}H-uridine (Fig. 10). In the tapetal cell, silver grains were typically localized over the nucleoli and the rest of the nuclear material, although in cells with intense incorporation, isolated silver grains were also found in the cytoplasm. In the endothecial cell, the first significant accumulation of silver grains occurred at nonvacuolate microspore stage. During the microspore stage, silver grain density was slightly above background. But after microspore mitosis, there was a sharp increase in ^{3}H-uridine incorporation (Fig. 11). Silver grain density was almost 3-4 times compared with microspore stage. Immediately after mitosis of the generative cell, the endothecium continued to incorporate the label at a reduced rate (Fig. 12). With the onset of radial thickening in the walls of the endothecial cells at mature 3-celled pollen stage, silver grains completely disappeared from these cells. In the endothecial cell, silver grains were found mostly in the nucleus, and sparsely in the surrounding cytoplasm. Apart from the tapetum and



Figs. 7-12. Autoradiographs showing incorporation of ^{3}H-uridine into cells of the anther wall at different stages of male gametogenesis. Fig. 7. Anther wall at the stage of microspore just after release from tetrads. Silver grains over cells of the anther wall are not found above the background. Fig. 8. Anther wall at the stage of microspore before vacuolation. Heavy incorporation of ^{3}H-uridine into the tapetal cells occurred. The label is found mostly in the nucleus. Silver grains also appeared at the epidermal and endothecial cells. Fig. 9. Anther wall at the partially vacuolate microspore stage. Silver grains are found in the cells of tapetum, endothecium and epidermis. The density of silver grains over the tapetal cells decreased. Fig. 10. Anther wall at the vacuolate microspore stage. Silver grains in the tapetal cell occurred only sparsely. Silver grains in the endothecial and epidermal cells appeared almost same degree as the anther at the partially vacuolate microspore stage. Fig. 11. Anther wall at the bicelled pollen stage. Dense incorporation of ^{3}H-uridine into the endothecial cells occurred. Silver grains also appeared at the epidermal cells. Fig. 12. Anther wall at the tricelled pollen stage. Silver grains appeared at the endothecial cells. (a) Tricelled pollen. (b) Same pollen, with focus on sperms (s). Calibration bar (20 μm) in Figure 7 applies to all the figures from 8 to 12.

endothecium, appreciable silver grains were found in cells of the epidermis from the nonvacuolate microspore stage to the bicelled pollen stage. During this period, autoradiographic silver grains over cells of the epidermis were present in more or less the same density and silver grain density was not high as compared with tapetum or endothecium. Silver grains over epidermal cells were found in the nucleus as well as in the cytoplasm. Most of the RNA labeled by ^{3}H-uridine in this experiment is presumed to be rRNA, since no chromosomal labeling was observed before nucleolar labeling.

As a test of the specificity of ^{3}H-uridine as a precursor of RNA synthesis in the cells of anther wall, representative slides were incubated in RNase (Sigma Chemical, 1 mg/mL in 0.05 M phosphate buffer, pH 6.0) for 24 h at 37℃ before applying the photographic emulsion. Absence of autoradiographic silver grains on the cells of anther wall pretreated with the enzyme as compared with anther pretreated with

the buffer alone for the same length of time confirmed that ^{3}H-uridine was actually being incorporated into RNA. The pattern of ^{3}H-uridine incorporation into cell of the anther wall of different stages was not altered when the incubation period of anthers in the isotope was extended to 4 h. The result indicates that failure of cells of the anther wall of specific stages of development to incorporate ^{3}H-uridine is not due to failure of the isotope to enter the anther tissues.

Since tapetal cells at microspore stage were binucleate, the number of silver grains per cell was not representative of the actual change in ^{3}H-uridine incorporation sites per set of diploid chromosomes. Nonetheless, the results indicate that cells of the anther wall become enriched with ^{3}H-uridine-incorporation sites at a specific stage of male gametogenesis. ^{3}H-uridine incorporation in tapetum was high at the nonvacuolate microspore stage while in endothecium it was high at the bicelled pollen stage. From these results, evidence has been presented for the existence of a stage-specific synthesis of RNA in cells of the anther wall of *B. napus* during male gametogenesis.

Among somatic tissues of the anther, most attention has been paid to the tapetum. Many experiments showed that the tapetum was critical for pollen development (Echlin, 1971; Goldberg *et al.*, 1993), and the pollen coat components, including lipids and proteins, were derived from the tapetum (Evans *et al.*, 1992). A stage-specific distribution of RNA has been observed in tapetal cells of *Hyoscyamus niger* (Raghavan, 1981; Raghavan and Jiang, 1992), *Lycopersicon esculentum* (Ursin *et al.*, 1989; Aguirre and Smith, 1993), tobacco (Koltunow *et al.*, 1990), rice (Raghavan, 1989) and *Brassica* (Shen and Hsu, 1992). The results of this work also showed the temporal expression of tapetal RNA. With minor variation, the expression pattern of RNA in *B. napus* tapetum was similar to that of those plants described above. RNA accumulation in *B. napus* tapetum was peaked at nonvacuolate microspore stage and decreased as the tapetal cells degenerated. Tapetal senescence begins at about microspore vacuolation. There appears to be a striking degree of coincidence between the peak accumulation of RNA and the periods of tapetal senescence, microspore vacuolation, and wall development on pollen grains. While these findings yield no informatin on the kind of RNAs, the present work shows that rather than passively secreting the contents of disintegrating cells into the anther loculus, the tapetum is actively engaged in the accumulation of informational macromolecules at a crucial stage in its ontogeny.

In contrast to the absence of silver grains in the tapetum after microspore mitosis, silver grains occurred in the endothecium. The finding that nucleus of the endothecial cell incorporate ^{3}H-uridine during pollen stage substantiates that the endothecial cell nucleus in pollen stage is biochmically functional. This is consistent with the fact that this cell type continue to differentiate even after microspore mitosis. In the case of the endothecium, this involves the formation of fibrous radial thickening.

The transient accumulation of RNA in cells of the anther wall of *B. napus* during male gametogenesis represents a novel observation which pinpoints the biochemical cytology of wall function closer than has been hitherto. But these results offer little guide as to which event is functionally related to RNA metabolism of the anther wall. Further studies aimed at identifying the kind of RNAs in the anther wall should provide a greater understanding of the role of these tissues during male gametogenesis and microspore embryogenesis.

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油菜의 雄性 配偶體 發生中 葯壁組織의 RNA 合成 變化

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摘 要

유채의 웅성 배우체 발생중 약벽조직의 RNA 합성 변화를 ^{3}H-uridine을 사용하여 방사선 자동사진법에 의해 조사하였다. 4분체기의 callose wall에서 방출된 직후 소포자기의 약벽 세포에는 silver grain이 background 이상 나타나지 않았다. 액포화가 일어나기 전 초기 소포자기에는 융단세포, 내피층세포 및 표피세포 모두에서 silver grain이 나타났다. 융단세포에서 silver grain이 나타나는 정도는 액포화 전 초기소포자기에 가장 높았으며 소포자의 액포화가 시작되는 시기에 융단조직이 붕괴되면서 감소되었고 2세포화분기에는 완전히 사라졌다. 내피층 세포의 RNA 축적은 소포자 분열 후 2세포화분기에 가장 높았으며 생식세포 분열 직후 초기 3세포화분기에도 비교적 높게 나타났다. 표피세포에서는 액포화가 시작되기 전 초기소포자기부터 2세포화분기까지 silver grain이 나타났는데 그 정도는 융단세포나 내피층세포에 비해 낮았으며 발달시기에 따른 차이가 거의 없었다. 3세포 성숙화분기의 약벽에는 silver grain이 나타나지 않았다. 약벽조직 대부분에서 silver grain은 주로 인과 핵에 나타났다.

주요어: 방사선 자동사진법, 유채, 웅성 배우체 발생, RNA 합성, 약벽

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