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Development of Pollen Grain Walls and Accumulation of Sporopollenin

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Abstract—By means of electron microscopy, we studied the development of pollen grain walls in *Calendula officinalis* L., *Dimorphotheca aurantiaca* DC., and *Cichorium intybus* L. (Asteraceae). As a reference, we studied the plants from the families Schisandraceae (*Schisandra chinensis* (Turcz.) Baill.), Lauraceae (*Persea americana* Mill.), Boraginaceae (*Borago officinalis* L.), and Cycadaceae (*Encephalartos altensteinii* Lehm.). In Asteraceae, we revealed two successively initiated layers of glycocalyx that form outer and inner layers of the ectexine. The formation of endexine is contributed by plasma membrane and small vesicles. Glycocalyx in the plants from the families Schisandraceae, Lauraceae, Boraginaceae, and Cycadaceae was found to consist of radially arranged helical cylindrical units, which are receptors of sporopollenin deposition. It is assumed that the receptor-independent accumulation of sporopollenin is also possible.

Key words: Schisandra chinensis - Borago officinalis - Persea americana - Encephalartos altensteinii - Calendula officinalis - Dimorphotheca aurantiaca - Cichorium intybus - glycocalyx - ectexine matrix - primexine - sporoderm - ectexine - endexine - sporopollenin

INTRODUCTION

In angiosperms, the formation of pollen grain wall (sporoderm) usually follows a common pattern. The sporoderm has several layers differing in the time of emergence, morphology, and the chemical composition [1, 2]. Its outer layer (exine) is subdivided into ect- and endexine and mainly consists of sporopollenin, the most stable biopolymer among organic materials, protecting the exine from the effects of acids, alkalis, high temperatures (up to 300°C) and ensuring its resistance to microorganisms. Monomers of sporopollenin are known to develop via oxidative polymerization of carotenoids and carotenoid esters in the cells of tapetum and partially in the pollen grain. The inner layer of the sporoderm (intine) may be lamellar; it does not contain sporopollenin and rapidly breaks down. The pollen grains of angiosperms are also notable for the external layer (tryphine), which usually emerges at the late stages of pollen formation within the anther cavity simultaneously with the intine. This layer is formed owing entirely to the activity of the cells of tapetum. The pollen grains are notable for apertures that are flexible areas of sporoderm, through which the pollen tubes penetrate and water and gas exchange occurs [3].

In spite of a unique nature of sporopollenin, a wide variety of the structure of exine in the taxa of seed plants, and application of the data about sporoderm structure in many fields, presently the formation of sporoderm remains a poorly investigated process. Temporary callose wall is the first to be formed around the developing microspores. This wall protects the initiation of glycocalyx, which occurs owing to the activity of cytoplasm. The glycocalyx is the ectexine matrix carrying the receptors capable of polymerizing the monomers of sporopollenin that arrive from the tapetum. At this time, only receptor-dependent sporopollenin is deposited on the matrix resulting in the formation of primexine that subsequently becomes the basis for the polymerization of receptor-independent sporopollenin that transforms primexine into ectexine. The way of sporopollenin monomer deposition on the glycocalyx may be detected only at the very early stages of the sporoderm development by means of high-resolution transmission electron microscope.

Using some representatives of plant families Asteraceae, Schisandraceae, Lauraceae, Boraginaceae, and Cycadaceae we described the successive stages of their sporoderm formation and sporopollenin accumulation in order to reveal general mechanisms and individual features of sporoderm formation in the investigated groups.

MATERIALS AND METHODS

Microspores and pollen grains were collected at different stages of development of *Schisandra chinensis* (Turcz.) Baill., *Persea americana* Mill., *Borago officinalis* L., *Encephalartos altensteinii* Lehm. (from the

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collections of Komarov Botanical Institute, Russian Academy of Science and the Botanical Gardens of Stockholm University), *Calendula officinalis* L. (Egor'evsk raion, Moscow oblast), *Dimorphotheca aurantiaca* DC. (the collection of the Botanical Gardens of Moscow State University), *Cichorium intybus* L. (Domodedovo raion, Moscow oblast) and fixed in 2.5% glutaric aldehyde at pH 7.3 (*C. officinalis*, *D. aurantiaca*, *C. intybus*) or in 3.0% glutaric aldehyde at pH 7.4 (*S. chinensis*, *P. americana*, *B. officinalis*, *E. altensteinii*) at room temperature for 24 h. Post-fixation was conducted in 2% OsO_4 for 3 h at room temperature (*S. chinensis*, *P. americana*, *B. officinalis*, *E. altensteinii*) or in 1% OsO_4 for 24 h at 4°C (*C. officinalis*, *D. aurantiaca*, *C. intybus*). Dehydrated material was embedded in the mixture of Epon resins, Epon-Araldite or the Spurr mixture. Ultrathin sections were prepared using an LKB microtome (Sweden), contrasted with uranyl acetate and lead citrate, and examined with a Hitachi H-600 and Jeol 100 B transmission electron microscopes (Japan).

RESULTS

Formation of the sporoderm starts as early as at the stage of mother cells around which temporary callose wall (Fig. 1a) is produced from the Golgi vesicles with a double-layer membrane and electron transparent contents. The vesicles approaching the plasmalemma often fuse, integrate with the membrane, and exude their contents outside of the cytoplasm (Figs. 1a, 1b). Thick transparent callose wall takes off the pressure between the neighboring microspores. Between the callose wall and plasmalemma, a primexine matrix (glycocalyx) arises (Figs. 1c, 1d, 1f, 1g), the basis for ectexine formation (Fig. 1h). This process is accompanied by the appearance of numerous elements of endoplasmic network and the Golgi apparatus, and emergence of Golgi vesicles (Fig. 1c). In the pollen grains of chicory, glycocalyx is formed identically (see Figs. 4a, 4b, 4c, 4e).

Figures 2a–2e show the formation of glycocalyx in the microspores of *Schisandra*. Fine-fibrillar layer of glycocalyx formed by the Golgi vesicles (Fig. 2a) appears to be thicker in the areas of plasmalemma invaginations, and on the tops of evaginations it becomes thinner. Here, electron dense columellae of ectexine produced as a result of sporopollenin polymerization are detected (Fig. 2a). By the middle of tetrad stage, these columellae increase in height and become more visible (Fig. 2b). By the end of tetrad stage, they become fungaceous (Fig. 2c). Between tetrad and post-tetrad stages, a thin foot layer emerges (Figs. 2d and 2e), and callose wall dissolves.

In *B. officinalis* (Figs. 2f–2j), glycocalyx consists of radial rod-shaped units (receptors of sporopollenin accumulation) forming columellae (Fig. 2f). In the middle of tetrad stage, the layer of primexine thickens, the columellae increase in height, and the elements of tectum emerge on the surface (Fig. 2g). In the base of the primexine, the foot layer emerges. At the beginning of free-spore stage, the foot layer, columellae, and discontinuous cover (tectum) are obvious. On the surface of the tectum, electron dense particles are formed (Figs. 2h, 2i). Subsequently, these particles are hardened to globules (gemmae) (Fig. 2j).

In the course of development of microspores in *E. altensteinii* (Figs. 1f, 1g) studied for comparing with the angiosperms, radial cylindrical units of glycocalyx were observed. Their surface is studded with electron dense particles (Fig. 1f). Such particles, the receptors of sporopollenin accumulation, become larger and more electron dense (Fig. 1g). Subsequently, this process leads to the formation of ectexine that consists of elongated alveoli.

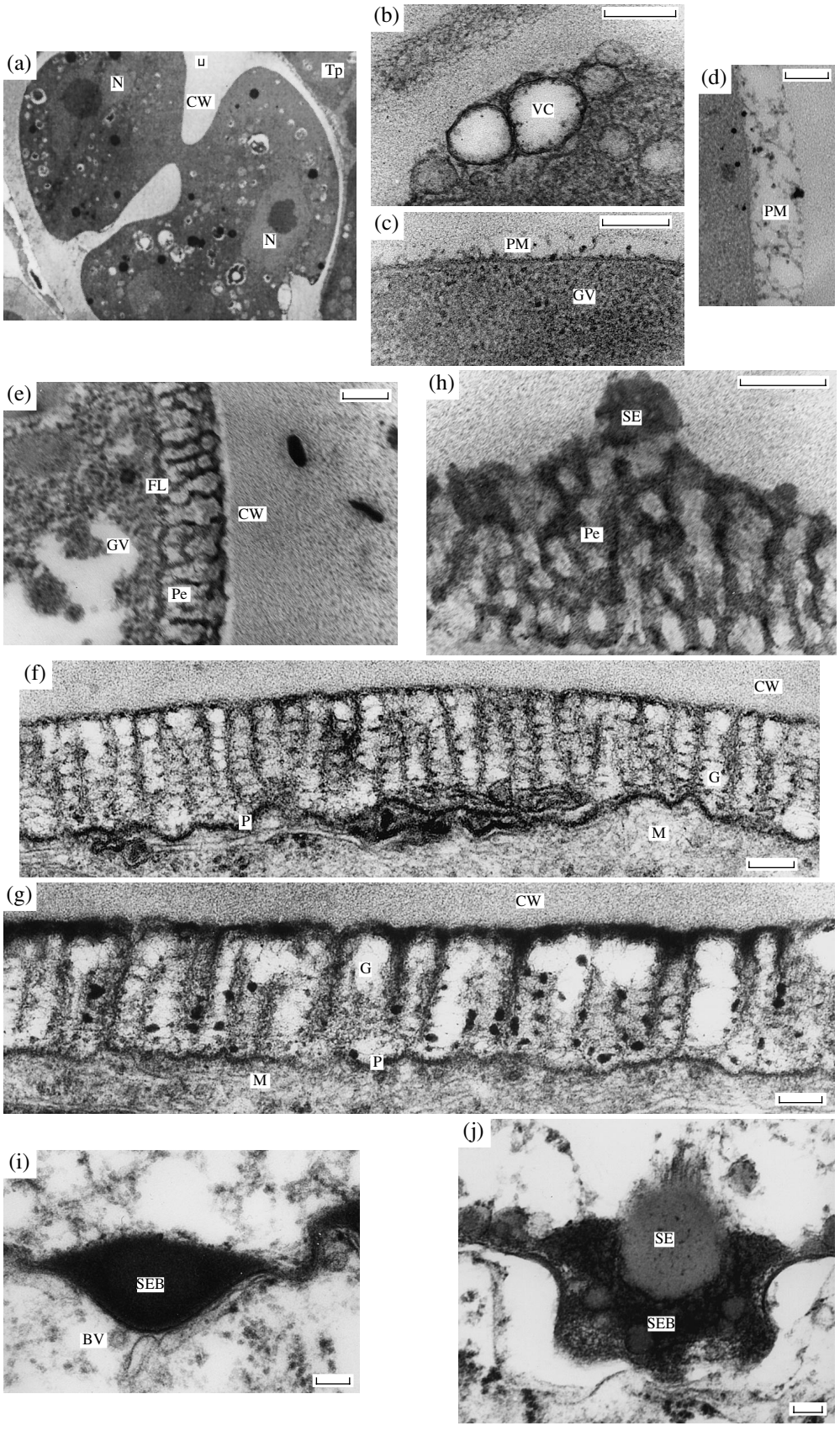
In *P. americana*, the exine consists of the sculptural elements and gemmae located among them. At the early stage of free spores, the beds of sculptural elements (Figs. 1i, 1j), and later the sculptural elements and gemmae, are formed in the invaginations of plasmalemma. When the beds of sculptural elements are already produced, round electron dense particles emerge on their surface and on the plasmalemma between them. These particles are similar to those formed in the course of exine development in *B. officinalis* and *E. altensteinii* (Fig. 2i). Subsequently, the clusters of these particles give rise to the sculptural elements, and between them, the gemmae are formed. Radial units of glycocalyx that were the basis for the

Fig. 1. Formation of exine in (a–h) *Calendula officinalis*, (f, g) *Encephalartos altensteinii*, and (i, k) *Persea americana*.

(a) Callose wall (tetrad formation); (b) callose wall formation (microspore); (c) beginning of the formation of primexine matrix (microspore); (d) formation of primexine matrix (microspore); (e) primexine matrix (middle tetrad stage); (f) development of glycocalyx (beginning of the middle tetrad stage); (g) formation of primexine matrix (middle tetrad stage); (h) formation of sculptural element (late tetrad stage); (i) formation of sculptural element (early free-spore stage); (j) formation of sculptural element (free-spore stage).

Bar is 0.2 μm.

Designations: A—aperture; AER—agranular endoplasmic reticulum; BV—bordered vesicle; C—cavity; Co—columella; Cr—crista; CW—callose wall; E—endexine; Ect—ectexine; EL—endexine lamellae; ER—endoplasmic reticulum; FL—foot layer; G—glycocalyx; GA—Golgi apparatus; GV—Golgi vesicles; I—intine; iI—intine I; iII—intine II; IC—internal columellae; ICE—internal cavity of ectexine; IME—internal matrix of ectexine; LG—lipid globule; M—microspore; N—nucleus; OC—outer columellae; P—plasmalemma; Pe—primexine; PM—primexine matrix; Pp—protoplast; SE—sculptural element; SEB—sculptural element bed; T—tectum; Tp—tapetum; VC—vesicles containing callose; VE—vesicles forming endexine.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formation of the sculptural elements are also observed here (Fig. 1h).

In all the Asteraceae examined, ectexine also develops on the glycocalyx produced from the vesicles of Golgi apparatus. However, in contrast to the plant species described earlier, the ectexine matrix in *Calendula* and *Dimorphotheca* is alveolate, and the columellae formed on such a matrix turn out to be alveolate inside (Figs. 1e, 1h, 3a, 3e). As a result of polymerization of sporopollenin monomers, the tectum, columellae, and foot layer are simultaneously formed on the ectexine matrix (Fig. 3e).

Another feature of ectexine formation in the Asteraceae is the formation under the outer ectexine of the second matrix of the inner ectexine (for example, Figs. 3a, 3b, 3c, 3e). However, the behavior of this matrix differs in various representatives. For instance, in *Calendula* sporopollenin is polymerized on the inner part of the matrix at the place of the discontinuous foot layer, and the upper part of the matrix transforms into a small cavity (Fig. 3d). *Dimorphotheca* is notable for the formation of cavities and a continuous foot layer (Figs. 3f, 3h). In *Cichorium* (Fig. 4), inner matrix forms large branched columellae under the cristae. Branching covers up to three fourth of the columellae height, and in the lower part rather large cavities are formed among the columellae (Figs. 4f, 4g). The sculptural elements that are formed on the cristae have inner cavities. The outer ectexine located between the cristae is thin and consists of thin external columellae, tectum, and foot layer (Figs. 4g, 4j); internal ectexine formed on the inner matrix of ectexine is represented by a narrow cavity of distinct width (Figs. 4g, 4j).

Endexine is formed after callose was dissolved at the post-tetrad stage. In all the examined species, endexine is produced on the basis of plasmalemma, which is repeatedly peeled off from the microspore cytoplasm (Figs. 3h, 4f, 4g, 4h, 4j). Between the layers of plasmalemma, a fine-fibrous interlayer is produced from the contents of vesicles entering from the microspore cytoplasm. These vesicles have a thin single-layer membrane (Fig. 4a). The sites of future apertures are visible at the very early stages of sporoderm formation, and glycocalyx is not produced at these sites (Fig. 4d).

In the Asteraceae, the bottom of apertures is formed at the time when the last lamellae of endexine are formed and consists of separate small globules that contain sporopollenin (Fig. 3g); intine is the main part of this bottom.

Intine and tryphine are formed at the stage of the pollen grain (Figs. 3d, 3g, 3h, 4i, 4j). At the initial stages, tryphine looks like separate electron dense and electron transparent vesicles and small granules (Figs. 4f, 4h, 4j). Somewhat later, the large elongated elements overlap them and finally all the structures fuse into a dense homogenous substance that fills the gaps (Fig. 4g).

DISCUSSION

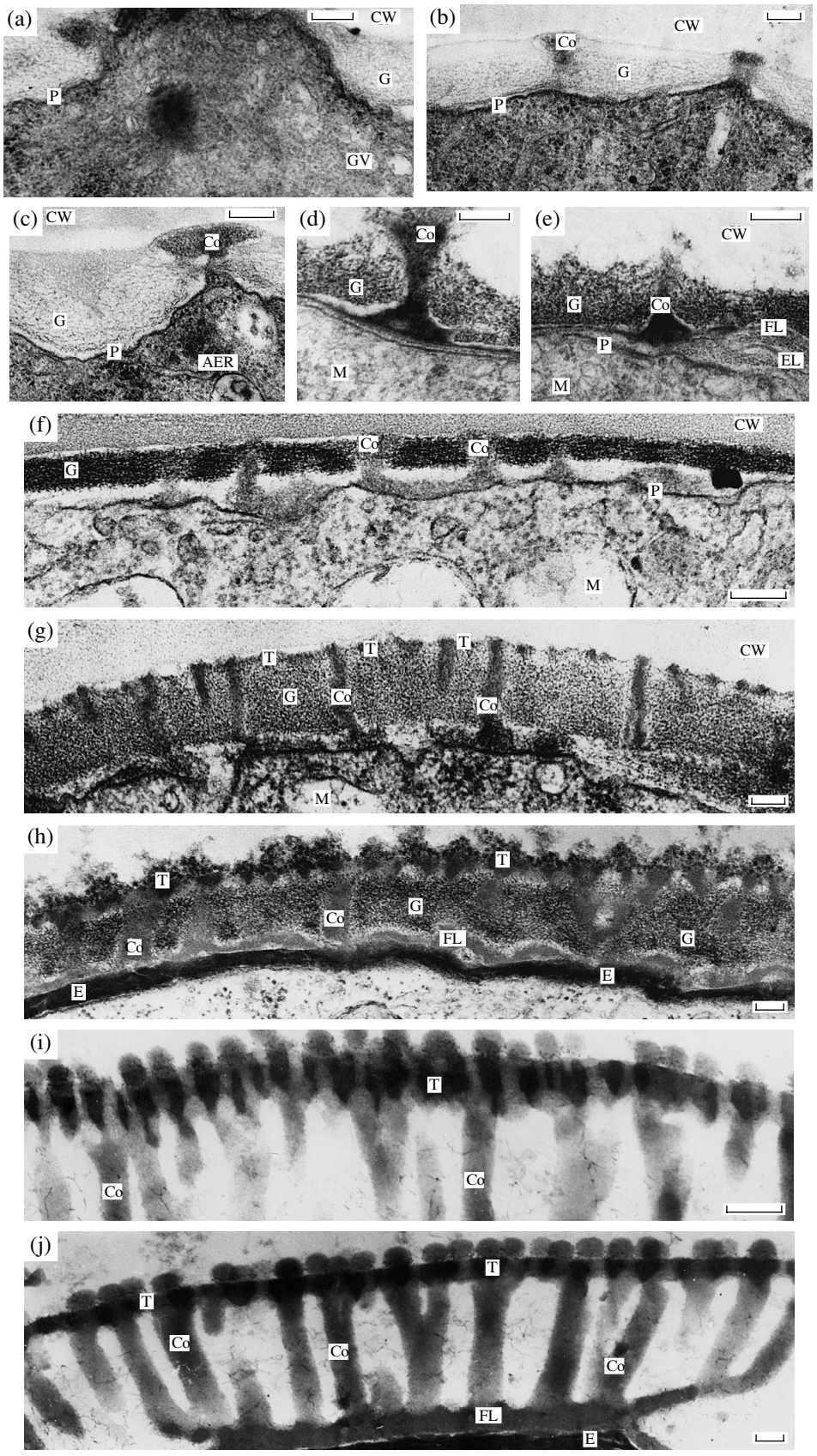
In this article, a special attention was paid to the formation of glycocalyx in the representatives of *Schisandra*, *Borago*, *Persea*, and *Encephalartos*. Glycocalyx, the matrix of primary ectexine, is a fibrillar three-dimensional network with hexagonal alveoli and spiral elements mainly oriented perpendicular to the plasmalemma. These two components of glycocalyx are more or less obvious in different species. For instance, in the course of development of exine in *S. chinensis* (Figs. 2b, 2c) we usually observed fibrillar glycocalyx, whereas in *B. officinalis* (Fig. 2g), *P. americana* (Figs. 1f, 1g), and *E. altensteinii* radial spiral elements were well discernible.

Our investigations corroborate the existence of spiral elements in the glycocalyx [5, 6] and suggest that the model of exine substructure proposed by Rowley [7] is correct. According to this model, glycocalyx is a system of complex tubular and spiral molecular complexes (Fig. 1f). For the most part, the elements of substructure are visible at the early tetrad stages before the onset of sporopollenin deposition that partially or completely masks it. However, the substructure is sometimes discerned in the mature parts of exine (for instance, see Fig. 2i). The diversity of exine patterns depends on the accumulation of sporopollenin on the glycocalyx. We put forward a hypothesis accounting for the determination of the exine design [8, 9]. Thus, in the course of exine formation in *B. officinalis*, *P. americana*, and *E. altensteinii*, we observed round electron dense particles that could be considered sporopollenin-accepting particles [10, 11]. They are protein-positive when tested with 5% phosphoarsenous acid in 10% acetone [11]. During tetrad stage, the exine pattern gradually develops and usually looks tender and low-contrast; following callose degradation, the exine becomes high contrasting. It was considered that after the disappearance of callose, the access of sporopollenin from tapetum to the microspore and subsequently to the pollen grain is considerably facilitated. However, now it becomes evident that callose does not hamper the penetration of sporopollenin precursors because it is the way of sporopollenin accumulation rather than its amount that is of importance.

Above, we considered the way of sporopollenin deposition on specific receptors. The experiments on

Fig. 2. Development of exine in *Schisandra chinensis* (a–e) and *Borago officinalis* (f–j).

(a) Early tetrad stage; (b) middle tetrad stage; (c) late tetrad stage; (d, e) free-spore stage; (f) early tetrad stage; (g) middle tetrad stage; (h) beginning of free-spore stage; (i) formation of tectum; (j) mature exine. The designations are the same as in Fig. 1.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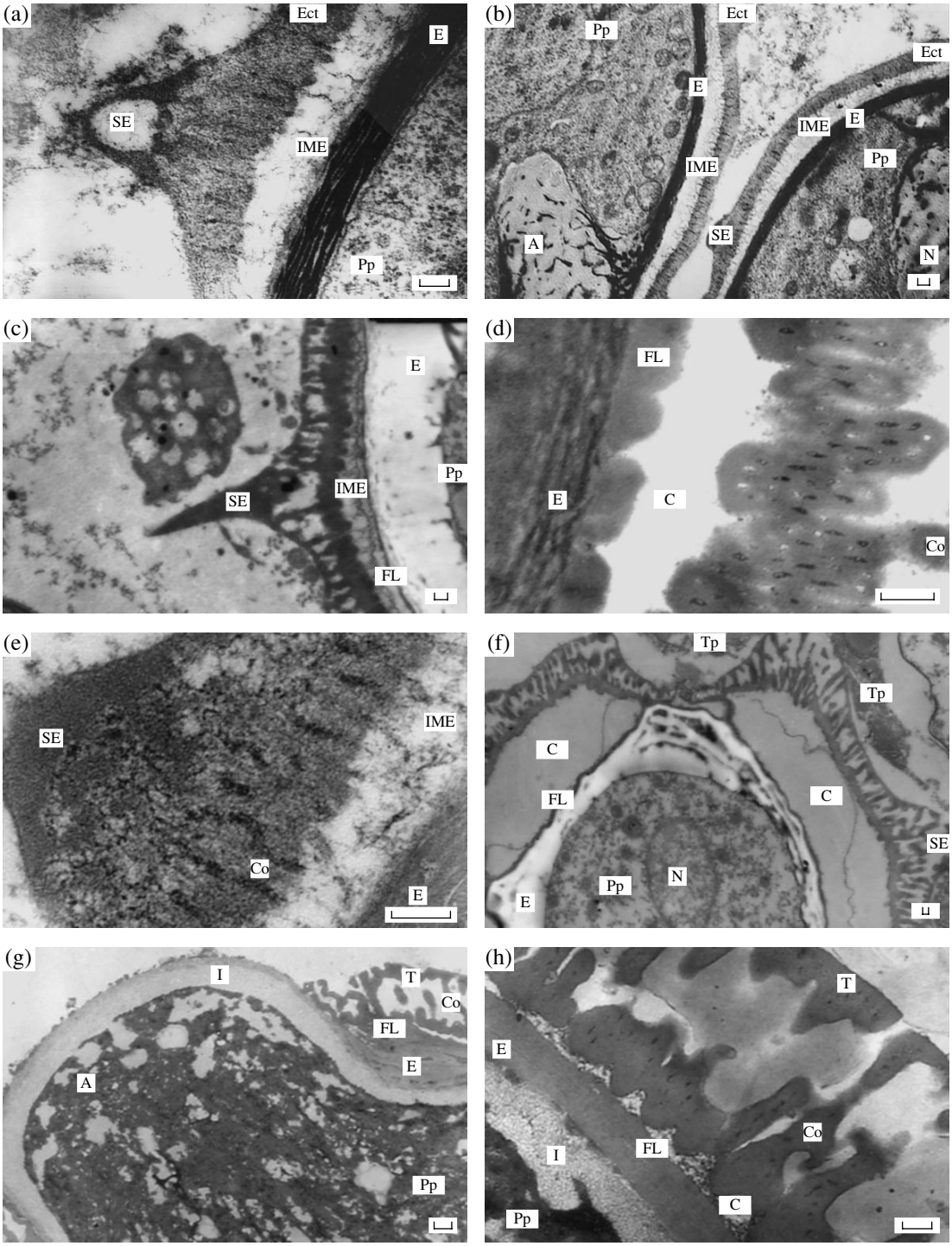

Fig. 3. Development of exine in *Calendula officinalis* (a–d) and *Dimorphotheca aurantiaca* (e–h).
(a) formation of sculptural element; (b) early post-tetrad stage;
(c) middle post-tetrad stage; (d) mature sporoderm; (e) formation of sculptural element; (f) formation of pollen grain; (g) aperture; (h) mature sporoderm. The designations are the same as in Fig. 1.

chemical and physical degradation of pollen grain walls suggested that sporopollenin deposition might also occur without the participation of receptors. Rowley and Claugher [12] showed considerable destruction of sporoderm following post-acetolytic treatment with potassium permanganate, which resulted in the exposing of structural units of the exine (tufts). Under scanning electron microscope of high resolution, these units

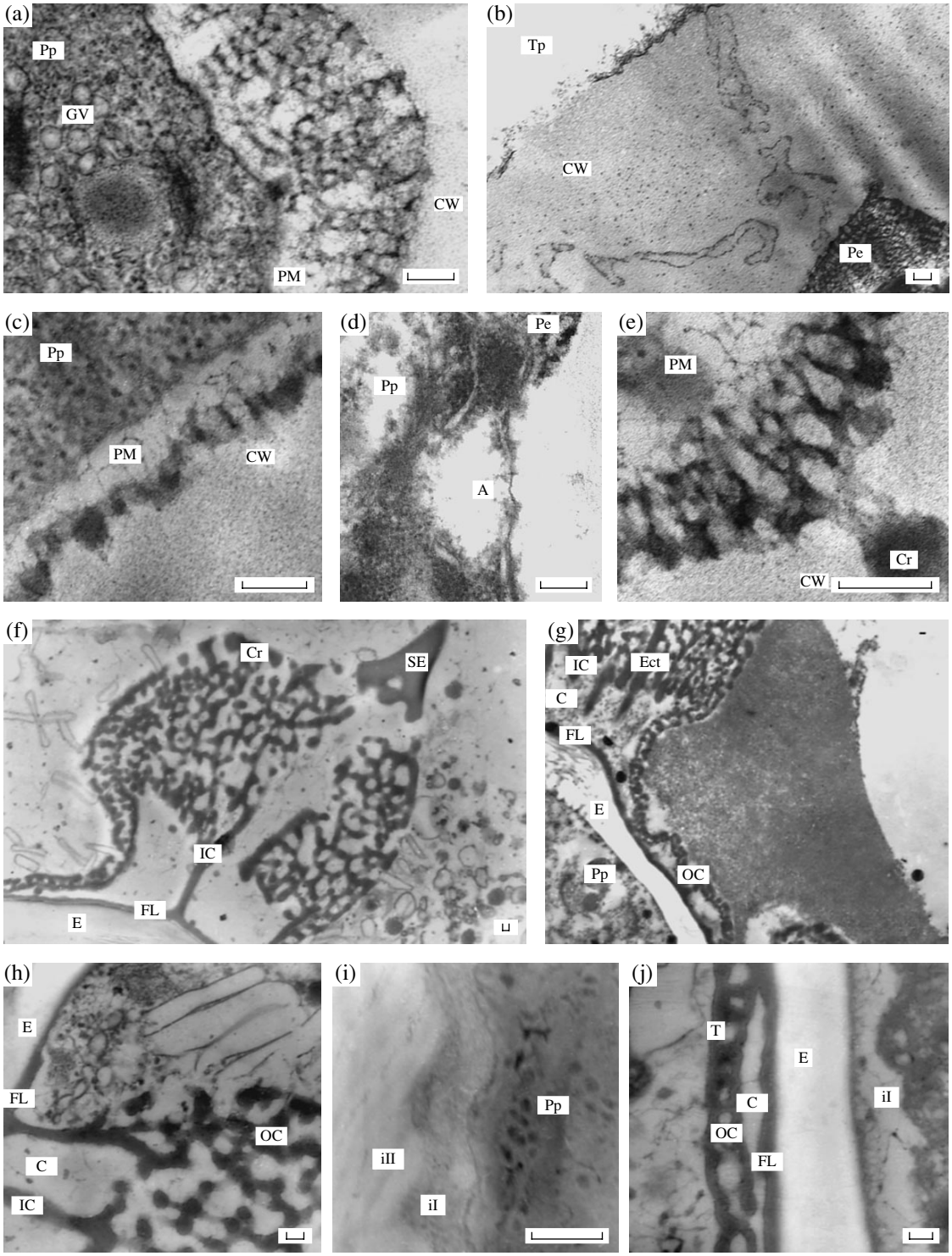

Fig. 4. Development of exine in *Cichorium intybus* L.
(a) Primexine matrix; (b) channels in callose wall ensuring the transfer of substances from tapetum to microspore; (c) lacuna without aperture; (d) lacuna with aperture; (e) formation of the crista; (f) crista; (g) lacuna without aperture and crista; (h) vesicles and granules in the cavity of the lacuna; (i) formation of the intine; (j) lacuna without aperture. The rest designations are the same as in Fig. 1.

look spiral (Fig. 1f). When pollen grains were bombarded after acetolysis with fast particles, the erosion of sporopollenin between substructural rod-shaped units was even more pronounced. These data suggest that sporopollenin accumulated directly on the subunits of the exine (receptors) is extremely resistant to destructive agents, whereas sporopollenin that accumulates later, fills the gaps between the tufts, and precipitates on

their surface, is more vulnerable to damaging agents. On the basis of these assumption, Rowley and Claugher [12] introduced the notions of primarily deposited (receptor-dependent) and secondarily deposited (receptor-independent) sporopollenin. The material presented in this article shows that in the course of the pollen development, round electron dense particles 10–15 nm in diameter are observed in the exine of different species. Apparently, these particles are receptor molecules accumulating primary receptor-dependent sporopollenin. Primary sporopollenin mainly accumulates during the tetrad stage at the beginning of the exine development. As a result, primexine emerges on the glycocalyx frame and accounts for the three-dimensional design of the exine architecture characteristic of a particular species. Subsequently, this fine construction is masked with great amount of secondarily deposited receptor-independent sporopollenin that apparently precipitates by means of self-assembly. The greatest part of sporopollenin in pollen grains probably consists of receptor-independent sporopollenin [13–15].

Evidence that sporopollenin is heterogeneous was available earlier. For instance, ectexine and endexine were shown to differ in resistance to 2-aminoethanol that partially dissolves sporopollenin and helps reveal its most vulnerable part and expose the substructure of exine [13]. Current biochemical investigations also show that sporopollenin consists of heterogeneous polymers [16–18] and its biosynthesis involves various reactions of secondary metabolism [19–21].

Out of all the investigated angiosperms, the formation and morphological structure of sporoderm of the Asteraceae turned out to be the most intricate [4, 22–27]. First of all, this feature manifests itself in the formation of two consequently formed layers of glycocalyx. As a result, two layers of ectexine that consist of columellae are produced in *Cichorium* (Figs. 4f and 4g) and *Artemisia* (according to Rowley [28]). In more special cases, the lower layer of ectexine preserves only foot layer (*Calendula*, Figs. 3c, 3d and *Dimorphotheca*, Figs. 3f, 3h), and the upper part of glycocalyx is incapable of polymerizing sporopollenin and forms cavities, sometimes spacious. The latter reduce the weight of the pollen grain and are the receptacles of fine-fibrillar substances that resemble intine in their appearance. Probably, together with the substances located on the surface of the pollen and between the columellae of ectexine, these substances may participate in the recognition of compounds released by the stigmata of the plants of the same species. Moreover, upon heavy moistening, these cavities may account for considerable increase in the volume of pollen grain cytoplasm without the rupture of its sporoderm.

In many Asteraceae, the structure of ectexine matrix is alveolate; as a result, inner spongy columellae are formed in *Calendula* (Figs. 1e, 1h, 3a). Dickinson and Potter described this phenomenon earlier in *Cosmos bipinnatus* [29]. Another characteristic of ectexine is the formation of intricately branched columellae; in the gaps between these columellae, numerous cavities containing sporopollenin arise (*Cichorium*, Figs. 4f, 4h). Thick multilayer pollen wall in Asteraceae is a light frame that retains sporopollenin and other substances inside numerous cavities. In this way, Asteraceae ensure not only mechanical but also biochemical protection of the contents of the male gametophyte.

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