Planta (1993) 189:139–149

Planta
© Springer-Verlag 1993

Cell-wall development in freeze-fixed pollen: Intine formation of *Ledebouria socialis* (Hyacinthaceae)

Michael W. Hess

Institut für Botanik und Botanischer Garten, Universität Wien, Rennweg 14, A–1030 Wien, Austria, FAX: 43 (1) 78 71 01 131

Received 27 March; accepted 2 August 1992

Abstract. The structure and development of the inner pectocellulosic pollen wall, the intine, was re-examined using high-pressure freezing with subsequent freeze substitution in *Ledebouria socialis* Roth, a monocotyledonous angiosperm. The bilayered intine is formed immediately after differentiation of the endexine. Similar to somatic cell walls, intine matrix substances originate from the Golgi apparatus and leave the cytoplasm via exocytosis. Exintine development starts with the apposition of intine matrix substances to the inner polysaccharide layer of the endexine (termed inner endexine), leading to irregular cell-wall ingrowths. Subsequently the inner endexine becomes intensely infiltrated with intine matrix substances; this process is interpreted as transformation of the inner endexine into intine. Along the aperture region, cell-wall matrix substances are unevenly deposited to such an extent that more or less radially oriented tubules filled with cytoplasm remain within the growing exintine. These tubules subsequently become cut off from the microspore cytoplasm by selective membrane fusions, leading to the incorporation of ground cytoplasm and ribosomes into the exintine. Exintine formation is completed prior to the first mitotic division of the pollen grain whereas the endintine is formed as a homogeneous thin layer after mitosis. Both transformation of the inner endexine by infiltration and passive incorporation of cytoplasm and ribosomes into the exintine by membrane fusions are novel features and are only observed in optimally freeze-fixed, freeze-substituted samples; general aspects of ultrastructure preservation in high-pressure-frozen, freeze-substituted plant cells are discussed as well. Modifications of the Golgi apparatus and post-Golgi-apparatus structures during pollen wall development are correlated with increasing and decreasing polysaccharide exocytosis, respectively. These events strictly coincide with the formation of morphologically and chemically different pollen wall layers and therefore seem to reflect the different deposition patterns of the predominant cell-wall polysaccharides.

Key words: Intine – Freeze substitution – Golgi apparatus – Pollen (high-pressure freezing) – *Ledebouria*

Abbreviations: ER = endoplasmic reticulum; FS = freeze substitution; HPF = high-pressure freezing; MS = microspore(s); PATAg = periodic acid-thiocarbohydrazine-silver proteinate; PGS = post-Golgi-apparatus structures; UA-Pb = uranyl acetate-lead

Introduction

The complex process of spore and pollen wall formation in vascular plants has attracted the attention of cell biologists since the light-microscopic work of Beer (1906). Since the 1950s, there have been many detailed electron-microscopic studies dealing mainly with the unique features of the outer pollen wall layer, the exine, which is largely composed of sporopollenins (Knox 1984; Southworth 1990). The findings of these studies indicate that several different patterns of exine formation exist within the spermatophytes (Dickinson 1976). In contrast, the inner pectocellulosic pollen wall layer, the intine, resembles primary walls of somatic tissues and is less variable, both in structure and development (reviewed by Heslop-Harrison 1979; Kress and Stone 1982). Research has therefore concentrated rather on histochemical characterization and biochemical analysis of intine substructures than on ontogenetic descriptions (reviewed by Knox et al. 1980; Knox 1984; Hesse 1991).

Recently introduced freeze-fixation methods are expected to provide additional information on pollen wall structure and development. In mycological ultrastructure research, for instance, rapid freezing techniques followed by freeze substitution (FS) have become routine during the last ten years. Their use has led to a partial revision of our knowledge of fungal ultrastructure (Hohl 1989; Hyde et al. 1991). In plant sciences, however, the impossibility of adequate freezing of large specimens, such as compact tissues, has until recently prevented the widespread adoption of freeze-fixation (Craig and Stae-

helin 1988). Now, high-pressure freezing (HPF) permits freeze-fixation of large specimens (up to 500 μm) without cryoprotection (Müller and Moor 1984; Studer et al. 1989). Hence, HPF and FS were chosen as state-of-the-art preparation methods for the ultrastructural investigation of intine development in *Ledebouria socialis* (Hyacinthaceae).

Material and methods

Specimen preparation. Plant material was grown in the greenhouses of the urban horticultural center (Stadtgärtnerei, Zürich, Switzerland). Freshly collected whole anthers of *Ledebouria socialis* Roth (Hyacinthaceae) were immersed in 1-hexadecene and kept for less than 1 min under mild vacuum to replace intercellular gases (Studer et al. 1989). The anthers were subsequently frozen in a high-pressure freezer (HPM 010; Balzers, Liechtenstein). Freeze substitution was performed in anhydrous acetone containing 2% OsO_4 using a Balzers FS unit (model FSU 010). The samples were kept at −90° C, −60° C and −30° C for 8 h at each step and finally brought to 0° C for 1 h. After rinsing three times in acetone the samples were infiltrated for 3 d with a graded resin series (Epon-Araldite) at room temperature. Sections were cut on a Reichert Jung Ultracut microtome (Leica, Vienna, Austria) with a diamond knife (Diatome, Biel, Switzerland), and were stained in an LKB UltroStainer (Leica) or by hand with uranyl acetate (40 min, 25° C) followed by lead citrate (5 min, 25° C) (UA-Pb staining). For better visualization of cell-wall details some of the sections were only briefly stained with UA-Pb (5 min each). Polysaccharides were localized on ultrathin sections collected on gold grids (Thiéry 1967): after removal of osmium with 10% H_2O_2 (30 min) sections were treated with 1% periodic acid (30 min), 0.2% thiocarbohydrazine (2 h) and 1% silver proteinate (30 min) at room temperature (PATAg staining). Controls were performed according to Courtoy and Simar (1973). The specimens were examined with an electron microscope (EM 109; Zeiss, Oberkochen, FRG) at 50 kV and 80 kV.

Terminology. Exine is subdivided into ectexine, made exclusively of sporopollenins, and endexine, consisting of sporopollenins and polysaccharides (Faegri and Iversen 1975; Knox 1984; Southworth 1990). Intine substrata are termed exintine and endintine according to Kress and Stone (1982).

Results

General observations. All cell types of the anther (i.e. microspores, tapetum, wall layers) were frozen without problems, irrespective of their developmental stage. The cell walls of different anther tissues appeared homogeneous, only occasionally showing microfibrillar substructures. All membranes and organelles appeared smooth and turgescent, respectively, and the plasma membrane was located adjacent to the cell wall. The contents of vacuoles and secretory vesicles were well preserved. Microfilaments were not observed during any of the developmental stages described.

Intine formation. Microspores (MS), and pollen grains, of *Ledebouria* have a single furrow-like germination region, the aperture, at the distal cell pole (Fig. 1).

After the release from the callosic special wall of the tetrads, young MS show two distinct cell-wall layers. Outermost sporopollenin elements, i.e. tectum, columellae and foot layer, form the ectexine (Figs. 1, 2). Between the foot layer and plasma membrane the young, undifferentiated endexine consists of a polysaccharide layer with sporadic sporopollenin lamellae embedded within (Fig. 2). At the aperture the ectexine consists of a thin foot layer only (Fig. 1) and the endexine does not contain any sporopollenin lamellae (Fig. 1). The plasma membrane is tightly appressed to the smooth inner surface of the endexine (Fig. 2).

The ground cytoplasm contains a few fairly small vacuoles and a moderate number of ribosomes; scattered profiles of endoplasmic reticulum (ER) occur throughout the cell (Fig. 1). Single microtubules are frequently closely associated with the plasma membrane, endomembranes or organelles (not shown). Dictyosomes consist of approximately five flat cisternae of different morphologies and staining patterns, i.e. a *cis*-to-*trans* polarity of the Golgi stack is evident. Membranes and the contents of Golgi cisternae are only moderately stained by UA-Pb (Fig. 2) or PATAg, respectively. Post-Golgi-apparatus structures (PGS; Mollenhauer and Morré 1991) are regularly found (Figs. 1, 2). Two types of vesicle are associated with the dictyosomes and PGS: darkly stained vesicles with a diameter of 70 nm and weakly stained ones with a diameter of 100 nm. Sometimes the small dark vesicles fuse with the plasma membrane, thereby discharging their contents into the endexine which gradually thickens (Fig. 2). More often,

Fig. 1. Young microspore of *Ledebouria socialis*. The cell wall consists of intensely stained sporopollenin elements of ectexine *(ex)* and endexine and a moderately stained homogeneous polysaccharide layer belonging to the endexine *(stars)*. At the aperture the ectexine consists only of a thin foot layer *(arrowheads)*. The cytoplasm contains only a few endomembranes and ribosomes. *n*, Nucleus; *d*, dictyosomes; *arrows*, PGS; *er*, endoplasmic reticulum; *v*, vacuoles; *m*, mitochondria; *p*, plastids. × 18 000; bar = 0.5 μm

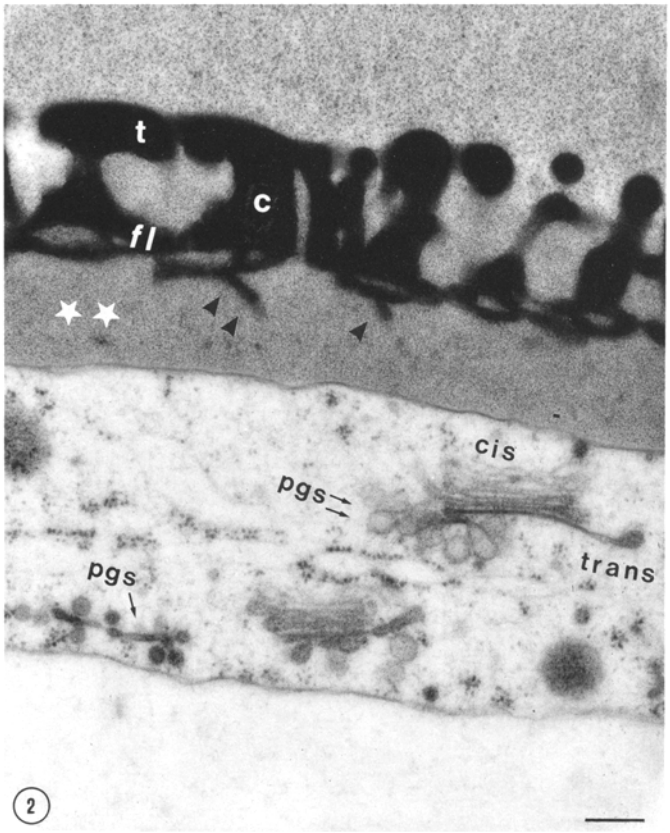
Fig. 2. Young MS of *Ledebouria*. Sporopollenin elements, i.e. tectum *(t)*, columellae *(c)*, foot layer *(fl)*, form the ectexine. The young, undifferentiated endexine consists of a homogeneous polysaccharide layer *(stars)* with scarce sporopollenin lamellae embedded within *(arrowheads)*. Dictyosomes show a clear *cis*-to-*trans* polarity; membranes and the contents of the cisternae are only moderately stained. Post-Golgi-apparatus structures *(pgs) (arrows)* are present in the vicinity of the *trans*-Golgi-face. Note the presence of two types of vesicle associated with dictyosomes and PGS. × 34 000; bar = 0.2 μm

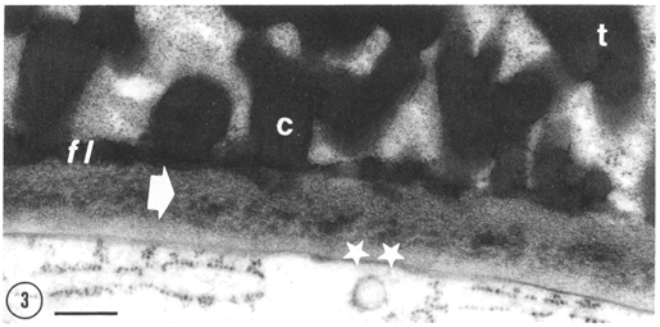
Fig. 3. Vacuolate MS of *Ledebouria*. The differentiated endexine consists of an outer layer intensely encrusted with sporopollenin *(large arrow)* and an inner homogeneous polysaccharide layer *(stars)*. Exine labelled as in Fig. 2. × 34 000; bar = 0.2 μm

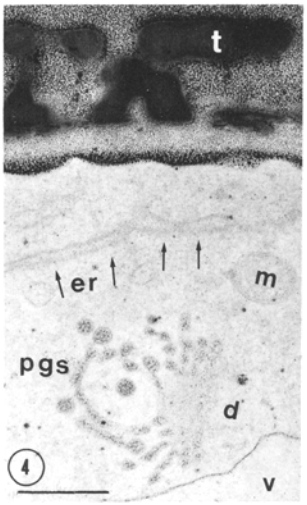
Fig. 4. Vacuolate MS; PATAg-reaction. The outer endexine layer, which is encrusted with sporopollenin, is weakly stained. In contrast, the inner endexine layer, which consists of polysaccharides as well as Golgi vesicles and PGS, is intensely stained. Exine and organelles are labelled as in Figs. 1, 2. × 23 000; bar = 0.5 μm

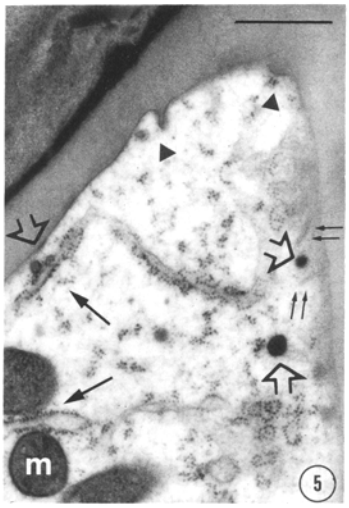
Fig. 5. Vacuolate MS (tangential section of the aperture region). Endoplasmic reticulum *(arrows)* and microtubules *(small arrows)* are located in close vicinity to the plasma membrane, Golgi vesicles *(open arrows)* and-or mitochondria *(m)*. Coated vesicles bud off the plasma membrane *(arrowheads)*. × 27 000; bar = 0.5 μm

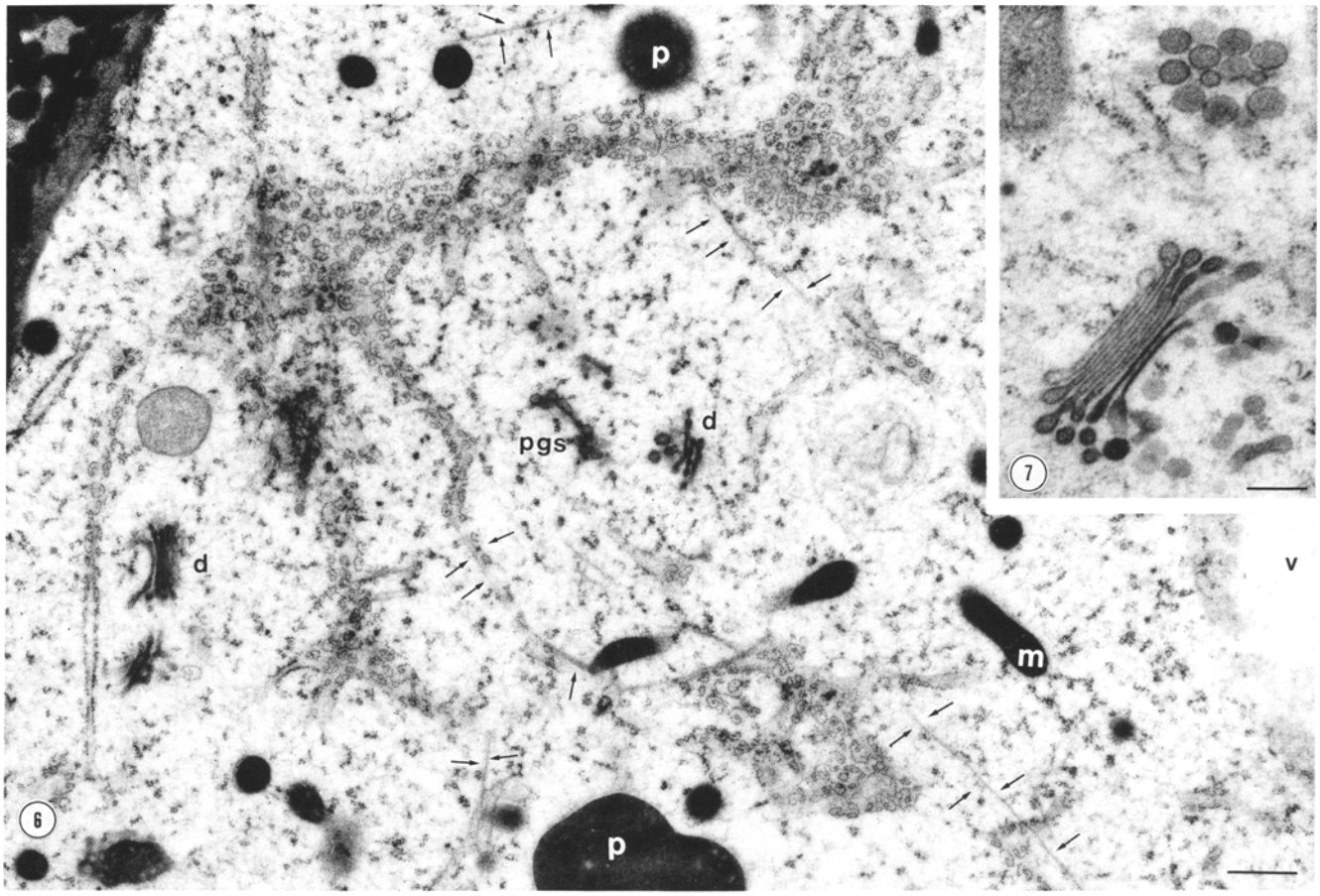

ex
d
v
d
p
d
m
p
er
n
1


t
c
fl
cis
pgs
trans
pgs
2

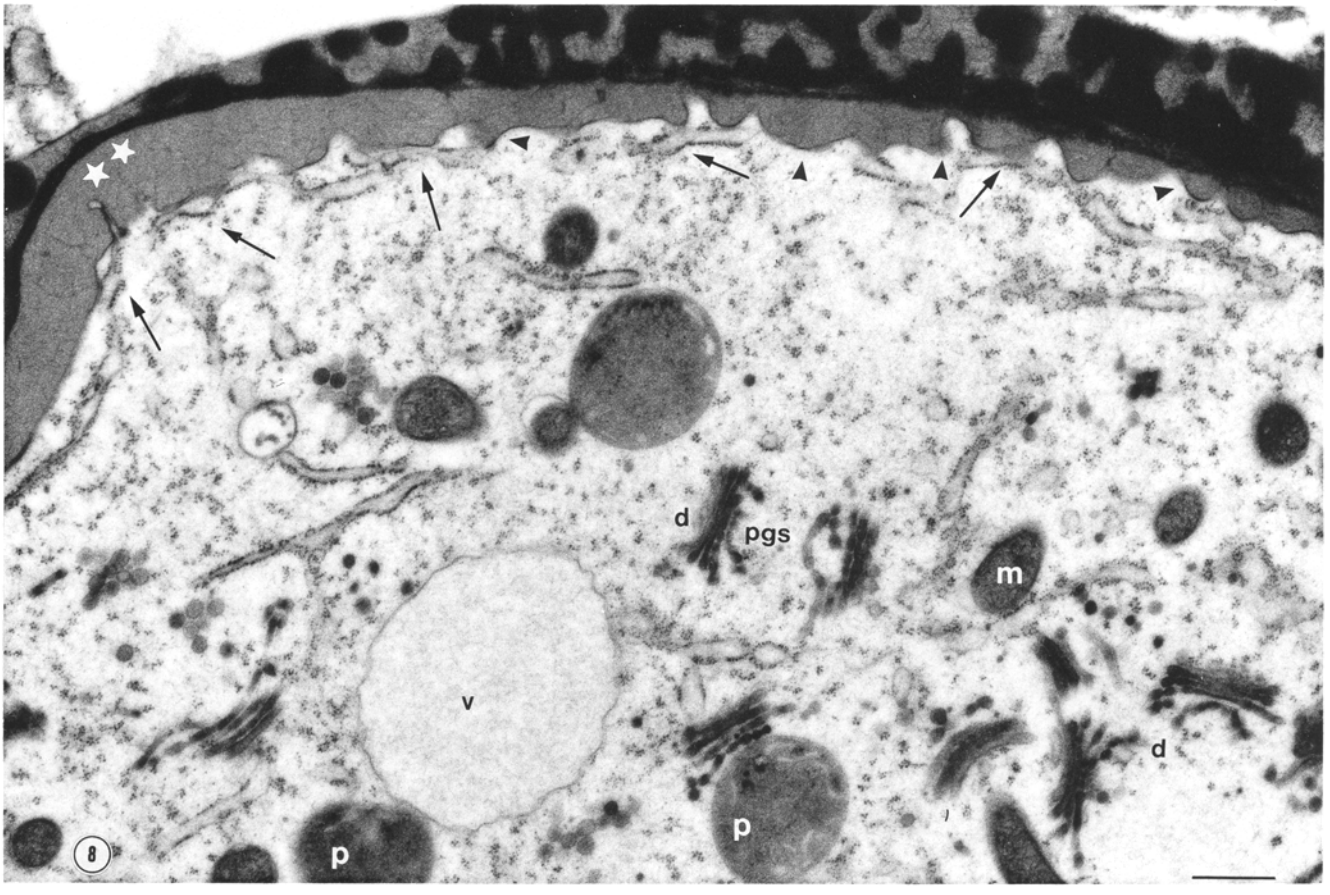

t
c
fl
3

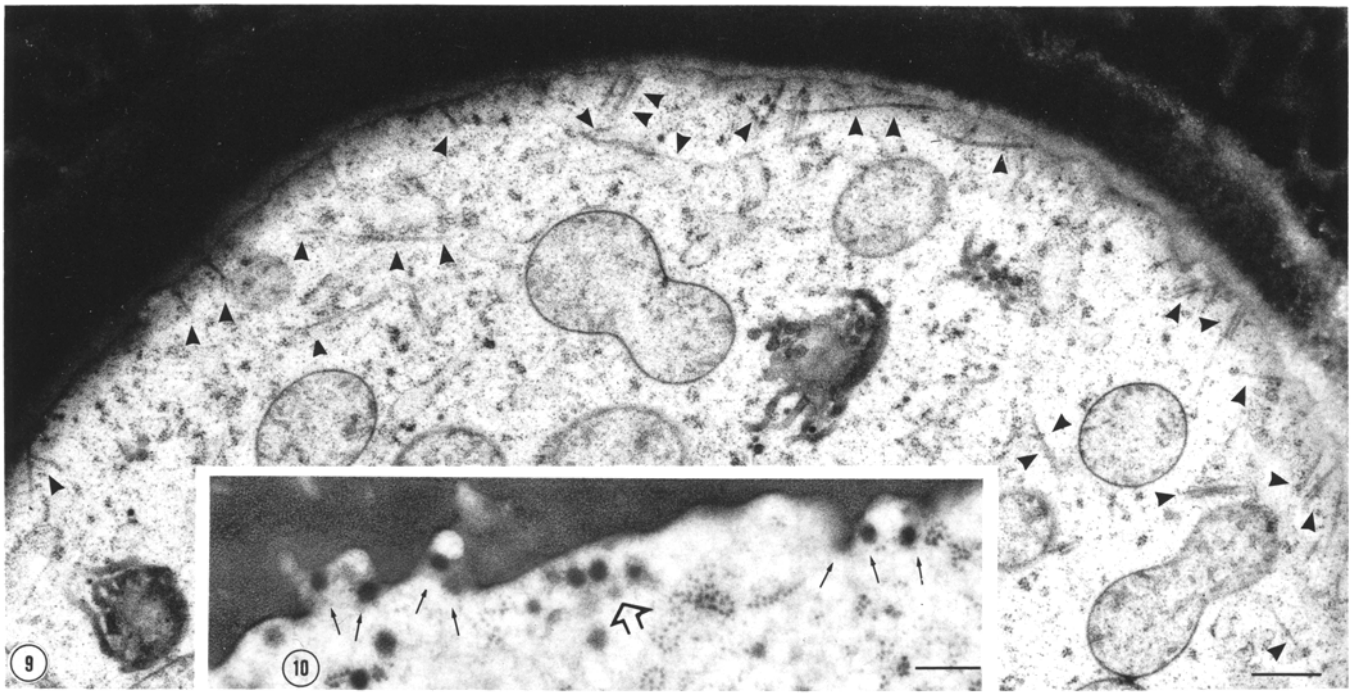

t
er
m
pgs
d
v
4


m
5


p
pgs
d
d
v
m
p
6

7


d
pgs
m
v
d
p
p
8



Figs. 9–10. Vacuolate MS of *Ledebouria socialis* during exintine formation

Fig. 9. Abundant microtubules *(arrowheads)* are present in the cell periphery, some of them in close vicinity to the plasma membrane (tangential section). × 19 000; bar = 0.5 μm

Fig. 10. Exintine matrix substances provided by Golgi vesicles leave the cytoplasm via exocytosis. Portions of ground cytoplasm are trapped by single or clustered Golgi vesicles fusing with the plasma membrane *(arrows)*. Some of the vesicles have a non-clathrin coat *(open arrow)*. × 46 000; bar = 0.2 μm

however, both kind of vesicle and-or PGS are seen in the vicinity of multivesicular bodies and-or small vacuoles (not shown).

Vacuolate MS show remarkable changes in both cell-wall and cytoplasmic ultrastructure. Two substrata are distinguishable within the endexine: the outer substratum is characterized by a coarse fibrillar polysaccharide network intensely encrusted with sporopollenin (Fig. 3), and it reacts only weakly to the PATAg test; the inner substratum (from now on termed inner endexine) is unchanged morphologically (Fig. 3) compared with the previous stage but is now strongly PATAg-positive (Fig. 4). Both substrata are unequally developed along the whole cell surface: at the aperture the inner substratum reaches its greatest thickness whereas the outer substratum is completely absent.

The cytoplasm of vacuolate MS is characterized by abundant ribosomes and ER which is frequently associated with microtubules (Fig. 6). Endoplasmic reticulum and microtubules frequently occur in close vicinity to the plasma membrane (Figs. 5, 8); this is also the case during the following developmental stages (Fig. 9). Dictyosomes have well-stained membranes, and the vesicles associated with dictyosomes and PGS are intensely stained with UA-Pb and PATAg (Figs. 4, 7, 8), indicating increased secretory activity when compared with the previous stage. Some of the Golgi vesicles have a coat morphologically different from a typical clathrin coat (Fig. 10, open arrow; see also Fig. 14).

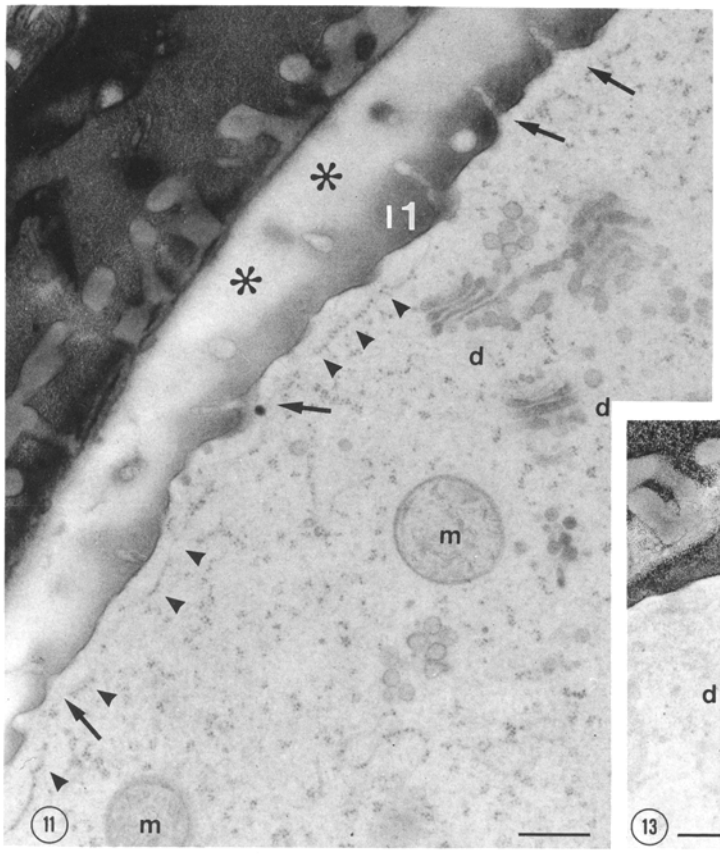
Exocytosis now mainly occurs at the aperture region, leading to irregular ingrowths of the cell wall (Fig. 8). This seems to mark the beginning of exintine formation. Interestingly, these cell-wall ingrowths are, at this time, not distinguishable from the inner endexine since the staining of both is moderately electron dense (Fig. 8).

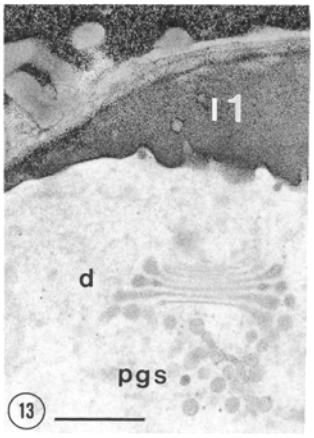
After the formation of cell-wall ingrowths the medium-stained inner endexine temporarily contrasts remarkably with the newly deposited, dark intine structures (Fig. 11); these staining properties are the only differences observable between the two transilient cell-wall zones.

Fig. 6. Vacuolate MS of *Ledebouria socialis* (tangential section). Abundant ER and associated microtubules *(arrows)* are seen in the cell periphery. Organelles labelled as in Fig. 1. × 18 000; bar = 0.5 μm

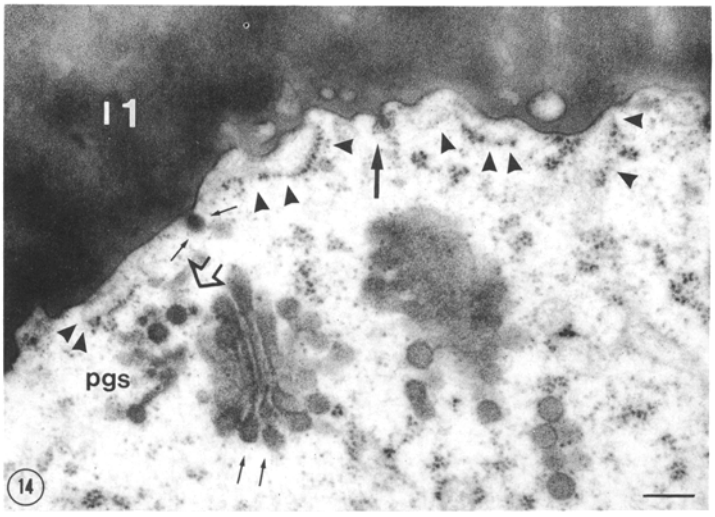
Fig. 7. Vacuolate MS at the beginning of intine formation. Dictyosomes have well-stained membranes; the associated vesicles are remarkably electron dense, indicating increased secretory activity of dictyosomes when compared with the previous stage. × 34 000; bar = 0.2 μm

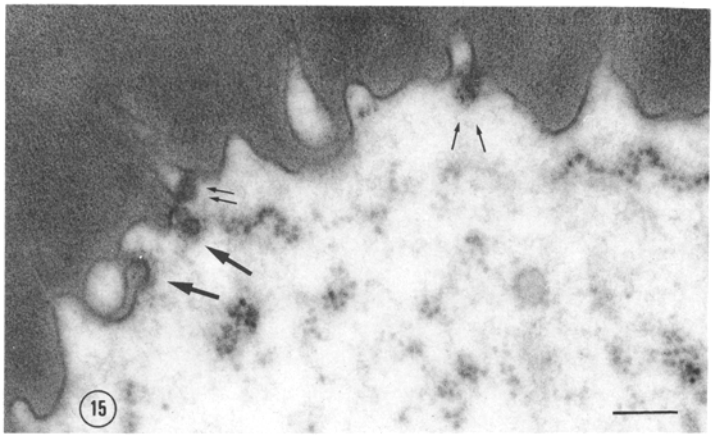
Fig. 8. Vacuolate MS at the beginning of intine formation. Exintine formation starts with the deposition of irregular cell-wall ingrowths at the aperture region. These exintine initials *(arrowheads)* and the inner endexine *(stars)* stain equally. Actively secreting dictyosomes *(d)* and PGS are intensely stained. Profiles of ER are located in close vicinity to the plasma membrane *(arrows)*. Organelles labelled as in Fig. 1. × 22 000; bar = 0.5 μm

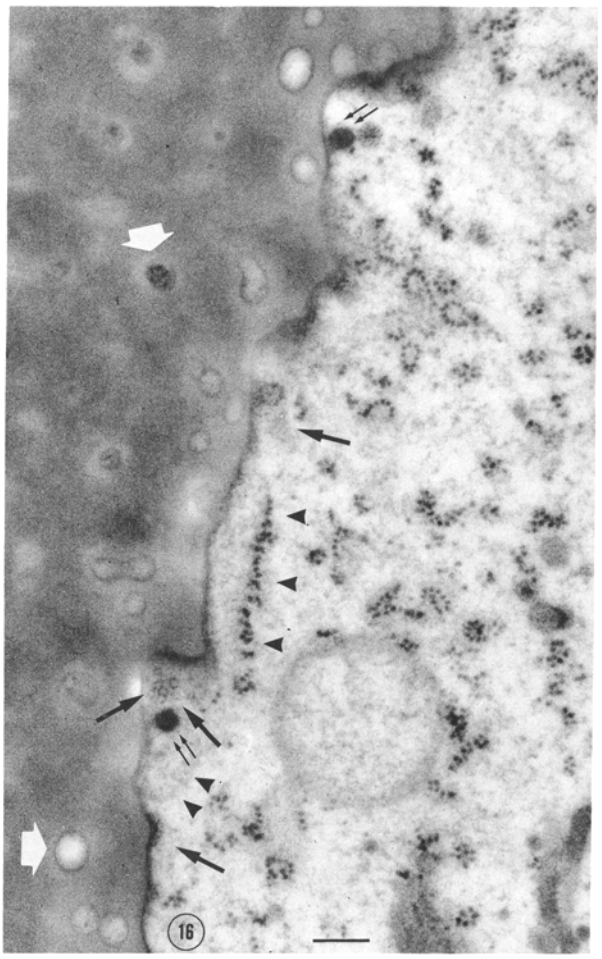

I1
d
d
m
m
11


I1
d
pgs
13

I1
12


I1
pgs
14


15


16

Subsequently, the inner endexine, too, becomes intensely stained and is no longer distinguishable from the exintine (Fig. 12). It seems as if the inner endexine really becomes infiltrated with intine matrix substances. Dictyosomes have densely stained cisternae producing large secretory vesicles, i.e. up to 100 nm in diameter (Figs. 11, 13, 14). Golgi vesicles with a coat morphologically different from a typical clathrin coat frequently accumulate at the *trans*-Golgi-face (Fig. 14). Membrane structures with a typical clathrin coat, i.e. coated pits and-or vesicles are regularly found at the cell wall-cytoplasm interface (Figs. 14, 15, 16).

Along the aperture region, cell-wall matrix substances are unevenly deposited to such an extent that more or less radially oriented tubules filled with cytoplasm remain within the growing exintine (Fig. 11). Those tubules subsequently become cut off from the MS cytoplasm (Figs. 10, 11, 15). The bases of the tubules are occluded (i) by single or clustered Golgi vesicles fusing with the plasma membrane or (ii) by fusions of plasma-membrane invaginations with each other. As a consequence of these membrane fusions, ground cytoplasm and ribosomes become incorporated into the growing exintine. Whether or not ER really becomes incorporated remained unclear, even after the analysis of serial sections, because optimal visualization of the ER requires a staining intensity different from that needed for the cell wall and plasma membrane, i.e. sufficient staining of ER with UA-Pb leads to overstaining of cell-wall details and the plasma membrane (e.g. Figs. 14, 16). The contents of the exintine tubules, i.e. cytoplasm and ribosomes, degrade soon after isolation from the MS cytoplasm. These protein inclusions within the exintine remain present until germination of the pollen grain, but they are sometimes barely visible when stained with UA-Pb alone (Fig. 18, 19).

Formation of the exintine is completed prior to the first mitotic division of the pollen grain. When complete, it consists of an extremely thickened "tubular" region at the aperture and a thin homogeneous layer at the non-apertural sites, both intensely stained by UA-Pb and PATAg (Figs. 13, 14). Concomitant with microspore and intine growth the foot layer ruptures at the aperture (not shown).

Endintine is formed after mitosis as a homogeneous layer of even thickness along the whole pollen-grain surface. Dictyosomes no longer appear hypertrophied, and their cisternae stain moderately (Figs. 17, 18, 19). Golgi vesicles providing endintine matrix material are small, i.e. 50–70 nm in diameter (Figs. 18, 19); PGS are abundant and consist of branched tubuli and-or cisternae and associated vesicles (Fig. 18). In strong contrast to the phase of exintine formation, ER profiles are now only occasionally found near the plasma membrane. Single and-or bundled microtubules are seen in close vicinity to the plasma membrane (Fig. 18). Endintine is completed before the detachment of the generative cell from the pollen-grain wall. Both intine strata now stain moderately; the following pollen wall layers can be distinguished: (i) ectexine (ii) lamellated endexine (iii) tubular exintine (with the integrated "inner endexine") (iv) homogeneous endintine.

Figs. 11–16. Vacuolate MS of *Ledebouria socialis* at the peak of exintine formation

Fig. 11. Cell wall during the transformation of inner endexine into exintine. The moderately stained inner endexine *(asterisks)* contrasts considerably with the newly deposited dark intine substances *(I 1)*. Cytoplasmic tubules within the growing exintine are marked by *arrows*. Note ER *(arrowheads)* in close vicinity to the plasma membrane. Brief UA-Pb staining (5 min each) for better visualization of cell-wall details. × 19 000; bar = 0.5 μm

Fig. 12. Cell wall after the transformation of inner endexine into exintine. The (former) inner endexine and exintine stain equally strongly and are not distinguishable from each other. The exintine *(I 1)* is considerably thickened at the aperture region. Brief UA-Pb staining (5 min each). × 19 000; bar = 0.5 μm

Fig. 13. PATAg staining. As well as the exintine *(I 1)*, the dictyosomes *(d)*, PGS and associated vesicles are intensely stained. × 23 000; bar = 0.5 μm

Fig. 14. Dictyosomes produce large secretory vesicles *(small arrows)* during exintine formation *(I 1)*. Some of the vesicles have a non-clathrin coat and are frequently seen at the trans-Golgi-face *(open arrow)*. A clathrin-coated vesicle is seen budding off the plasma membrane *(arrow)*. The ER *(arrowheads)* is located in close vicinity to the plasma membrane. × 34 000; bar = 0.2 μm

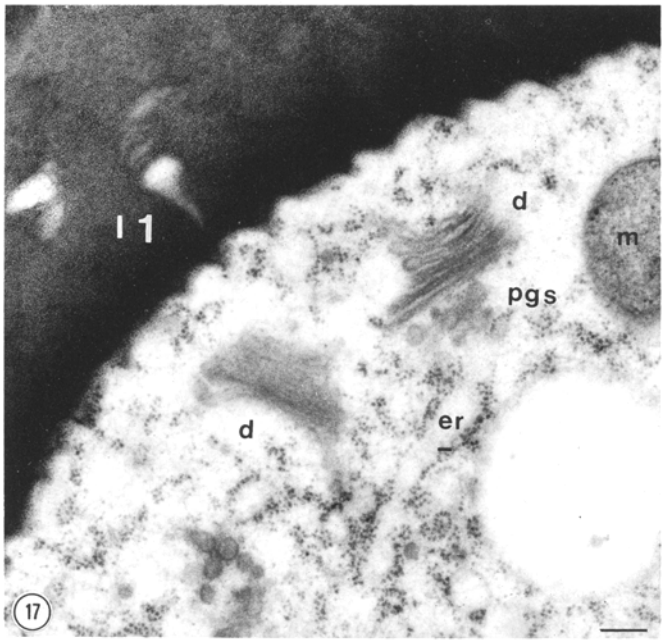
Fig. 15. Coated vesicles *(arrows)* are budding off the plasma membrane. Secretory vesicles *(small arrows)* appear to occlude the bases of cytoplasmic tubules within the exintine. × 46 000; bar = 0.2 μm

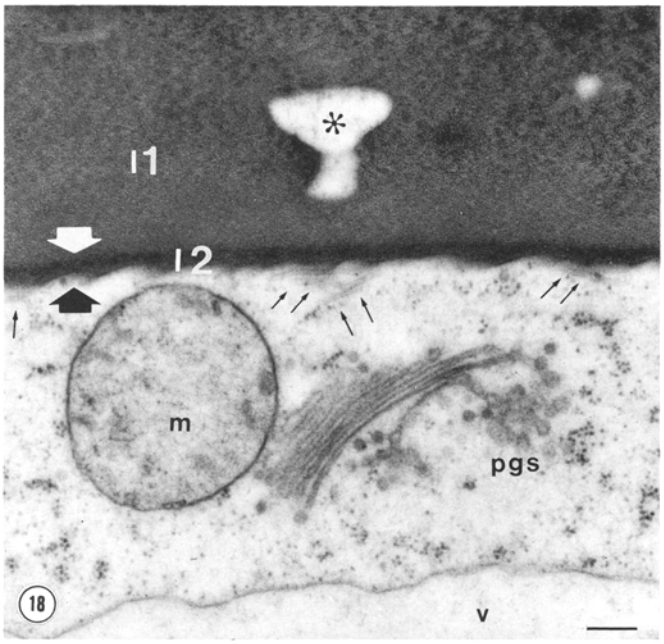
Fig. 16. Aperture region in tangential section. Coated pits *(arrows)* are present at the cell wall-cytoplasm interface. Secretory vesicles are marked with small arrows. The ER *(arrowheads)* is located in close vicinity to the plasma membrane. Radially oriented cytoplasmic tubules within the exintine appear in cross-section *(white arrows)*. × 46 000; bar = 0.2 μm

Discussion

Ultrastructure preservation in general. High-pressure freezing and freeze substitution have been shown to be well suited for the preparation of microspores that react sensitively to chemical fixation (Heslop-Harrison 1968). These two processes yield superior ultrastructure preservation of microspores compared with conventional chemical fixation, as has already been demonstrated for rapidly frozen, freeze-substituted mature pollen grains and pollen tubes (e.g. Cresti et al. 1990; Noguchi 1990). High-pressure freezing requires a minimum of preparation prior to freezing so that intact anthers are frozen immediately after sampling without any cryoprotective treatment. Thus the delicately balanced conditions within the anther are neither mechanically nor chemically disturbed. It is therefore assumed that the risk of inducing artifacts as a consequence of traumatic and/or osmotic reactions within the cells is negligible.

Microfibrillar cell-wall substructures are barely visible in high-pressure frozen, freeze-substituted anther tissues of *Ledebouria,* which is consistent with findings on freeze-substituted transfer cells (Browning and Gunning 1977). By contrast, cell walls in cryosections (apple





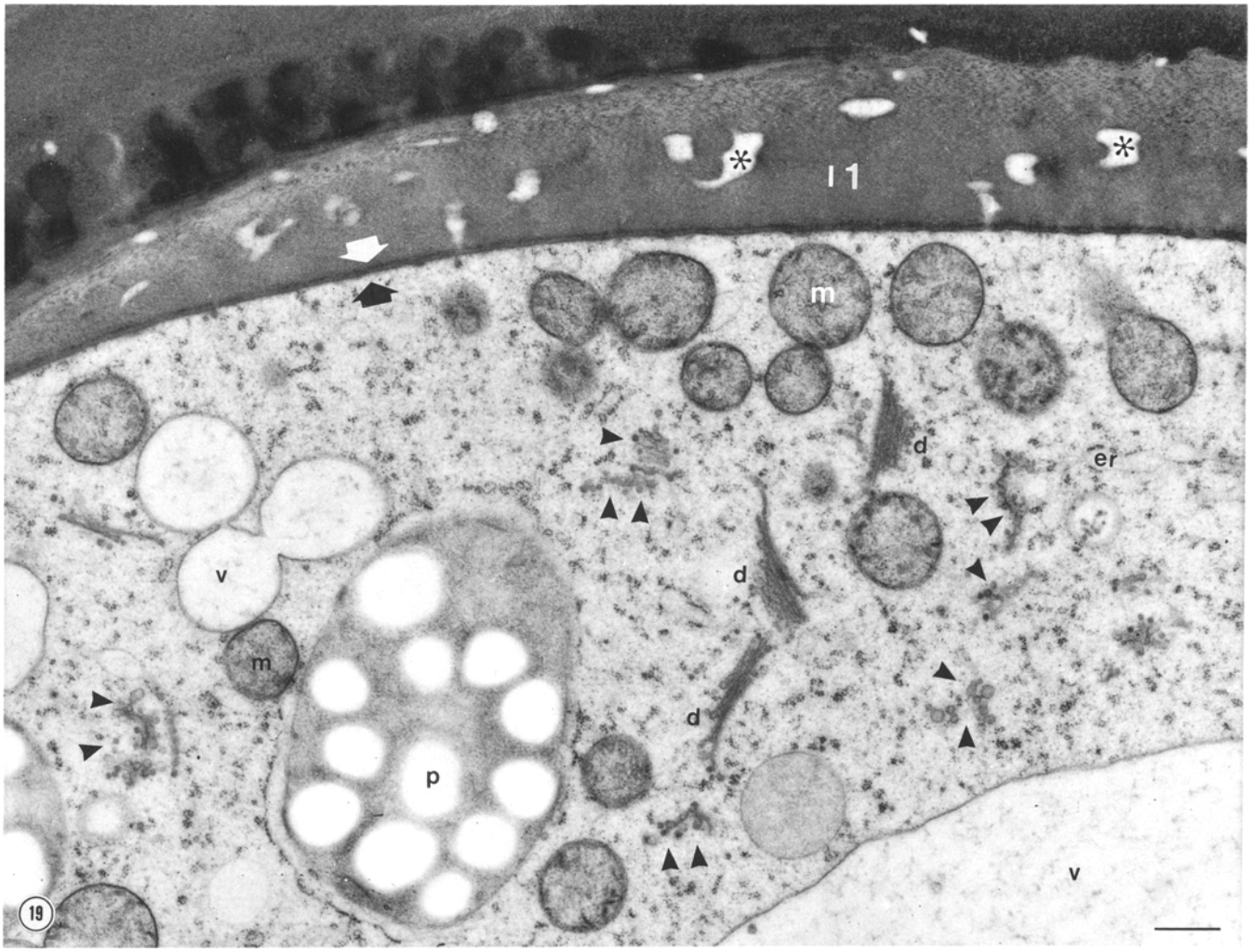


Fig. 17. Microspore of *Ledebouria socialis* prior to mitosis. Exintine *(I 1)* is completed. Dictyosomes *(d)* are no longer hypertrophied and stain moderately. × 34 000; bar = 0.2 µm

Figs. 18, 19. Young pollen grain during endintine formation. Endintine *(I 2)* is formed as a homogeneous thin layer *(large arrows)*. Dictyosomes *(d)* have moderately stained cisternae; PGS *(arrowheads)* consist of branched tubules and associated vesicles. Vesicles associated with dictyosomes and PGS are small (50–70 nm in diameter). Microtubules in close vicinity to the plasma membrane are marked by *small arrows;* "tubular or vesicular inclusions" within the exintine *(I 1)* are marked by *asterisks*. Organelles labelled as in Fig. 1. **Fig. 18** × 34 000; bar = 0.2 µm. **Fig. 19** × 18 000; bar = 0.5 µm

leaves, Michel 1991) show clearly discernable microfibrils even though the contrast of cryosections is in general far below that of material which has been freeze-substituted, resin-embedded and then stained with heavy metals.

An other interesting phenomenon is the obvious absence of microfilaments in *Ledebouria,* although the presence of actin microfilaments in angiosperm MS and young pollen grains has been demonstrated by rhodamine-phalloidin staining (Van Lammeren et al. 1989). Ding et al. (1992) recently reported adverse effects of HPF on the preservation of microfilaments; furthermore, OsO_4 is known to destroy actin filaments (Maupin-Szamier and Pollard 1978). Other observations, however, prove the excellent preservation of microfilament bundles in plant cells which have been frozen under high pressure and freeze-substituted in acetone containing OsO_4 (e.g. Lichtscheidl et al. 1990). Hence, it seems more likely that microfilaments in MS and/or young pollen grains occur only as single microfilaments which are not sufficiently stained by the applied substitution and staining protocol.

Classification and terminology of pollen wall layers. Intine is generally bilayered in monocotyledons (Kress and Stone 1982). Nakamura (1979), however, reported three intine layers in *Lilium,* classifying all polysaccharide layers of the pollen wall as intine. In *Ledebouria* such a classification is not possible: it has been shown that the homogeneous polysaccharide layer in MS of *Ledebouria* (termed young, undifferentiated endexine) becomes at least partly encrusted with sporopollenin; sporopollenin deposition during microsporogenesis in spermatophytes is restricted to exine development (Knox 1984; Southworth 1990).

Intine formation. Intine development in *Ledebouria* starts immediately after endexine differentiation, with exocytosis and irregular apposition of Golgi-synthezised cell-wall substances to the inner, polysaccharide stratum of the endexine (i.e. inner endexine). Similiar cell-wall ingrowths have been documented by Rowley and Erdtman (1967) and Argue (1971) but the former interpreted those structures as belonging to the endexine ("nexine 2"). Because of the superior preservation quality made possible by HPF-FS, it could be demonstrated that the inner endexine subsequently becomes infiltrated with intine matrix substances, i.e. transformed into exintine. This interpretation based on morphological observations is supported by histochemical findings: prior to infiltration, Alcian-Blue staining for polyanions such as pectins (Scott et al. 1964) and treatment with pectinase and hemicellulase have no visible effect on the inner endexine. After infiltration, however, the former inner endexine stains intensely with Alcian Blue and, like the exintine, is strongly affected by enzymic digestion (data not shown). Interestingly, a gradual transition zone between exintine and (former) endexine as well as the above-reported transformation process is only observed in optimally frozen samples. In contrast, specimens with a segregation pattern (i.e. freezing artifacts), as well as chemical fixed material, show distinct cell-wall strata. This phenomenon may be due to different chemical properties of the two ontogenetically different wall zones.

Exocytosis of Golgi-synthezised matrix substances is an ubiquitous process during growth of plant cell walls (Morré and Mollenhauer 1976; Willison 1981). Unequal exocytosis and deposition of cell-wall material often leads to the formation of irregular wall ingrowths with cytoplasmic tubules left between the individual ingrowths. Such wall structures have been reported, for instance, for a variety of transfer cells in vascular plants (Gunning and Pate 1969; Schnepf and Pross 1976; Browning and Gunning 1977). The isolation of cytoplasmic tubules leading to the incorporation of cytoplasm, ribosomes and probably also ER into the cell wall, however, is restricted to exintine formation. "Tubular/vesicular protein inclusions" of different, but more or less specific size, shape and distribution within the exintine have been found throughout the angiosperms (Rowley 1960; Heslop-Harrison 1979) and their functions in pollen germination and self incompatibility have been elucidated as well (Knox et al. 1980). They have in part been recognized as enclosed cytoplasm and "ribosome-like particles" (Christensen and Lersten 1972; Pacini and Juniper 1979; Fernandez and Rodriguez-Garcia 1989), respectively, but detailed information on the incorporation mode is limited. Nakamura (1979) reported that incorporation of "contents of the endoplasmic reticulum" within the intine seems to result from "the fusion of the endoplasmic reticulum with the cell membrane". Preparation by HPF-FS yielded superior preservation of structures apparently associated with the incorporation process: in *Ledebouria* ground cytoplasm, ribosomes and probably also parts of the ER appear to be trapped within the cell wall as a result of selective membrane fusions. Analogous structures, i.e. coated plasmamembrane invaginations fusing with each other, have been observed during the development of so-called "charasomes" (Lucas and Franceschi 1981). Coated membrane invaginations, i.e. coated pits, which are not directly related to the formation of specialized cell-wall structures are regularely observed at the cell wall-cytoplasm interface of *Ledebouria* MS as well. Similar coated-membrane configurations have been reported for various conventionally and freeze-fixed plant and animal cells (Browning and Gunning 1977; Ryser 1979; Emons and Traas 1986; Staehelin and Chapman 1987; Foissner 1988; Craig and Staehelin 1988; Lin et al. 1991; Emons and Vos 1992) and have partly been postulated to be involved in membrane recycling (e.g. Emons and Traas 1986 and references therein; Staehelin and Chapman 1987). Although the use of HPF-FS revealed novel structural details of exintine formation, further investigations of the cell wall-cytoplasm interface are necessary to elucidate these complex membrane fusion and-or recycling events in detail. It remains unclear, for instance, how the very selective membrane fusions which lead to the different, more or less specific patterns of exintine tubules within different systematic groups of angiosperms are brought about. Regulative functions in cell-wall formation and similar secretory processes are, in general, attributed to the ER (Hepler 1982; Steer 1988; Hepler et al.

1990 and references therein); this may also be the case in exintine formation where abundant ER in the vicinity of the plasma membrane is a common feature (Knox and Heslop-Harrison 1970; Nakamura 1979; Gabarayeva 1986).

The configuration of the Golgi stack and PGS, and its modification during intine formation in *Ledebouria* agrees quite well with reports on growing root tips (Mollenhauer et al. 1961; Kiss et al. 1990; Staehelin et al. 1990). In *Ledebouria,* changes in the morphology and activity of the Golgi apparatus and in the configuration of the plasma membrane strictly coincide with the deposition of morphologically and chemically different pollen wall layers: during exintine formation dictyosomes are hypertrophied and the plasma membrane appears undulated as a result of intense polysaccharide exocytosis. Exintine generally consists of Golgi-derived cell-wall matrix substances and is devoid of cellulose and callose (Kress and Stone 1982). In contrast, during endexine and endintine growth, dictyosomes are not hypertrophied and the outlines of the plasma membrane are smooth. Both strata contain only few matrix substances: endintine is mainly composed of cellulose (Kress and Stone 1982; Knox 1984) whereas information on the exact nature of endexine polysaccharides is limited (Knox 1984). Vithanage and Knox (1979), for instance, identified callose within the endexine of sunflower pollen. Cellulose as well as callose are assumed to be synthezised at the plasma membrane (Delmer 1987). It is therefore assumed that modifications of Golgi-apparatus and plasma-membrane configurations during pollen wall development of *Ledebouria* reflect the different deposition patterns of the dominant cell-wall carbohydrates of the endexine, exintine and endintine. With regard to the non-clathrin coat (Orci et al. 1986) of secretory vesicles in plant cells, further investigations are necessary, as pointed out by Kiss et al. (1990) who have already observed such vesicles in root tip cells subjected to HPF-FS.

I am grateful to Dr. Martin Müller (Institut für Zellbiologie, ETH-Zürich) for the kind permission to use the high-pressure freezer and the freeze-substitution unit at his laboratory. I wish to thank Prof. M. Hesse, Mag. M.G. Schlag (Institut für Botanik, Universität Wien) and Dr. I. Lichtscheidl (Institut für Pflanzenphysiologie, Universität Wien) for helpfull discussions. Thanks are also due to A. Glaser and W. Urbancik for excellent technical assistence and to the Stadtgärtnerei Zürich for providing the plant material. This work was supported by the Austrian "Fonds zur Förderung der wissenschaftlichen Forschung".

References

Argue, C.L. (1971) Pollen of the Butomaceae and Alismataceae. I. Development of the pollen wall in *Butomus umbellatus* L. Grana **11,** 131–144

Beer, R. (1906) On the development of the pollen grain and anther of some Onagraceae. Beih. Bot. Zbl. (Erste Abt.) **19,** 286–313

Browning, A.J., Gunning, B.E.S. (1977) An ultrastructural and cytochemical study of the wall-membrane apparatus of transfer cells using freeze-substitution. Protoplasma **93,** 7–26

Christensen, J.R., Lersten, N.R. (1972) Pollen wall and tapetal orbicular wall development in *Sorghum bicolor* (Gramineae). Am. J. Bot. **61,** 604–623

Courtoy, R., Simar, L.J. (1973) Importance of controls for the demonstration of carbohydrates in electron microscopy with the silver methenamine or the thiocarbohydrazide-silver proteinate methods. J. Microsc. **100,** 199–211

Craig, S., Staehelin, L.A. (1988) High pressure freezing of intact plant tissues. Evaluation and characterization of novel features of the endoplasmic reticulum and associated membrane systems. Eur. J. Cell Biol. **46,** 80–93

Cresti, M., Milanesi, C., Salvatici, P., Van Aelst, A.C. (1990) Ultrastructural observations of *Papaver rhoeas* mature pollen grains. Bot. Acta **103,** 349–354

Delmer, D.P. (1987) Cellulose biosynthesis. Annu. Rev. Plant Physiol. **38,** 259–290

Dickinson, H.G. (1976) Common factors in exine deposition. In: The evolutionary significance of the exine, pp. 67–89, Ferguson, I.K., Muller, J., eds. Academic Press, London

Ding, B., Turgeon, R., Parthasarathy, M.V. (1992) Effect of high-pressure freezing on plant microfilament bundles. J. Microsc. **165,** 367–376

Emons, A.M.C., Traas, J.A. (1986) Coated pits and coated vesicles on the plasma membrane of plant cells. Eur. J. Cell Biol. **41,** 57–64

Emons, A.M.C., Vos, J.M. (1992) Exocytosis in embryogenic suspension cells of carrot. Acta Bot. Neerl. **41,** 105

Faegri, K., Iversen, J. (1975) Textbook of pollen analysis, third edn., Munksgaard

Fernandez, M.C., Rodriguez-Garcia, M.I. (1989) Developmental changes in the aperture during pollen grain ontogeny in *Olea europaea* L. New Phytol. **111,** 717–723

Foissner, I. (1988) The relationship of echinate inclusions and coated vesicles on wound healing in *Nitella flexilis* (Characeae). Protoplasma **142,** 164–175

Gabarayeva, N.I. (1986) Ultrastructure analysis of the intine development of *Michelia fuscata* (Magnoliaceae) in connection with the changes of cytoplasmic organelles of microspores and tapetum. [In Russ., Engl. summary.] Both. Zh. (Leningrad) **71,** 416–428

Gunning, B.E.S., Pate, J.S. (1969) "Transfer cells". Plant cells with wall ingrowths, specialized in relation to short distance transport of solutes – their occurrence, structure and development. Protoplasma **68,** 107–133

Hepler, P.K. (1982) Endoplasmic reticulum in the formation of the cell plate and plasmodesmata. Protoplasma **111,** 121–133

Hepler, P.K., Palevitz, B.A., Lancelle, S.A., McCauley, M.M., Lichtscheidl, I. (1990) Cortical endoplasmic reticulum in plants. J. Cell Sci. **96,** 355–373

Heslop-Harrison, J. (1968) Some fine structural features of intine growth in the young microspore of *Lilium henryi.* Port. Act. Biol. Ser. A **10,** 235–246

Heslop-Harrison, J. (1979) Aspects of the structure, cytochemistry, and germination of the pollen of rye (*Secale cereale* L.). Ann. Bot. **44** (suppl. 1), 1–47

Hesse, M. (1991) Cytology and morphogenesis of pollen and spores. Prog. Bot. **52,** 21–34

Hohl, H.R. (1989) Cytology and morphogenesis of fungal cells. Prog. Bot. **51,** 1–20

Hyde, G.J., Lancelle, S., Hepler, P.K., Hardham, A.R. (1991) Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora,* a result with implications for cytokinesis in other eukaryotes. J. Cell Sci. **100,** 735–746

Kiss, J.Z., Giddings, T.H., Staehelin, L.A., Sack, F.D. (1990) Comparison of the ultrastructure of conventionally fixed and high pressure frozen/freeze substituted root tips of *Nicotiana* and *Arabidopsis.* Protoplasma **157,** 64–74

Knox, R.B. (1984) The pollen grain. In: Embryology of angiosperms, pp. 197–271, Johri, B.M., ed. Springer, Berlin Heidelberg New York Tokyo

Knox, R.B., Heslop-Harrison, J. (1970) Pollen wall proteins: Localization and enzymic activity. J. Cell Sci. **6,** 1–27

Knox, R.B., Vithanage, H.I.M.V., Howlett, B.J. (1980) Botanical immunocytochemistry: a review with special reference to pollen antigens and allergens. Histochem. J. **12,** 247–272

Kress, W.J., Stone, D.E. (1982) Nature of the sporoderm in monocotyledons, with special reference to pollen grains of *Canna* and *Heliconia*. Grana **21,** 129–148

Lichtscheidl, I.K., Lancelle, L.A., Hepler, P.K. (1990) Actin-endoplasmic reticulum complexes in *Drosera:* Their structural relationship with the plasmalemma, nucleus, and organelles in cells prepared by high pressure freezing. Protoplasma **155,** 116–126

Lin, H.Ch., Morre, M.S., Sanan, D.A., Anderson, R.G.W. (1991) Reconstruction of clathrin-coated pit budding from plasma membranes. J. Cell Biol. **114,** 881–891

Lucas, W.J., Franceschi, V.R. (1981) Characean charasome-complex and plasmalemma vesicle development. Protoplasma **107,** 255–267

Maupin-Szamier, P., Pollard, T.D. (1978) Actin filament destruction by osmium tetroxide. J. Cell Biol. **77,** 837–852

Michel, M. (1991) Kryo-Ultramikrotomie hochdruckgefrorener pflanzlicher und mikrobieller Proben: ein Vergleich mit konventionellen Kryo-Präparationsverfahren. Ph.D. thesis, Federal Institute of Technology, Zürich

Mollenhauer, H.H., Morré, D.J. (1991) Perspectives on Golgi apparatus form and function. J. Electron Microsc. Tech. **17,** 2–14

Mollenhauer, H.H., Whaley, W.G., Leech, J.H. (1961) A function of the Golgi apparatus in outer rootcap cells. J. Ultrastr. Res. **5,** 193–200

Morré, D.J., Mollenhauer, H.H. (1976) Interaction among cytoplasm, endomembranes, and the cell surface. In: Encyclopedia of plant physiology, N.S., vol. 3: Transports in plants III, Intracellular interactions and transport processes, pp. 288–344, Stocking, C.R., Heber, U., eds. Springer, Berlin Heidelberg

Müller, M., Moor, H. (1984) Cryofixation of thick specimens by high pressure freezing. In: The science of biological specimen preparation, pp. 131–138, Revel, J.P., Barnard, T., Haggins, G.H., eds. SEM, AMF O'Hare, Chicago

Nakamura, S. (1979) Development of the pollen grain wall in *Lilium longiflorum*. J. Electron Microsc. **28,** 275–284

Noguchi, T. (1990) Consumption of lipid granules and formation of vacuoles in the pollen tube of *Tradescantia reflexa*. Protoplasma **156,** 19–28

Orci, L., Glick, B.S., Rothman, J.A. (1986) A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. Cell **46,** 171–184

Pacini, E., Juniper, B.E. (1979) The ultrastructure of pollen grain development in the olive, *Olea europaea*. 1. Proteins in the pore. New Phytol. **83,** 157–163

Rowley, J.R. (1960) The exine structure of "cereal" and "wild" type grass pollen. Grana Palynol. **2,** 9–15

Rowley, J.R., Erdtman, G. (1967) Sporoderm in *Populus* and *Salix*. Grana Palynol. **7,** 518–567

Ryser, U. (1979) Cotton fiber differentiation: occurrence and distribution of coated and smooth vesicles during primary and secondary wall formation. Protoplasma **98,** 223–239

Scott, J.E., Quintarelli, G., Dellovo, M.C. (1964) The chemical and histochemical properties of Alcian Blue. I. The mechanism of Alcian Blue staining. Histochemie **4,** 73–85

Schnepf, E., Pross, E. (1976) Differentiation and redifferentiation of a transfer cell: Development of septal nectaries of *Aloe* and *Gasteria*. Protoplasma **89,** 105–115

Southworth, D. (1990) Exine biochemistry. In: Microspores. Evolution and ontogeny, pp. 193–212, Blackmore, S., Knox, R.B., eds. Academic Press, London San Diego New York Boston Sydney Tokyo Toronto

Staehelin, L.A., Chapman, R.L. (1987) Secretion and membrane recycling in plant cells: novel intermediary structures visualized in ultrarapidly frozen sycamore and carrot suspension-culture cells. Planta **171,** 43–57

Staehelin, L.A., Giddings, T.H., Kiss, J.Z., Sack, F.D. (1990) Macromolecular differentiation of Golgi stacks in root tips of *Arabidopsis* and *Nicotiana* seedlings as visualized in high pressure frozen and freeze-substituted samples. Protoplasma **157,** 75–91

Steer, M.W. (1988) The role of calcium in exocytosis and endocytosis in plant cells. Physiol. Plant. **72,** 213–220

Studer, D., Michel, M., Müller, M. (1989) High pressure freezing comes of age. Scanning Microsc. **3,** 253–259

Thiéry, J.P. (1967) Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. J. Microscopie **6,** 987–1018

Van Lammeren, A.A.M., Bednara, J., Willemse, M.T.M. (1989) Organization of the actin cytoskeleton during pollen development in *Gasteria verrucosa* (Mill.) H. Duval visualized with rhodamine-phalloidin. Planta **178,** 531–539

Vithanage, H.I.M.V., Knox, R.B. (1979) Pollen development and quantitative cytochemistry of exine and intine enzymes in sunflower, *Helianthus annuus*. Ann. Bot. **44,** 95–106

Willison, J.H.M. (1981) Secretion of cell wall material in higher plants. In: Encyclopedia of plant physiology, N.S., vol. 13B: Plant carbohydrates II, pp. 513–541, Tanner, W., Loewus, F.A., eds. Springer, Berling Heidelberg