Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10084–10088, October 1995
Plant Biology

Pathway of terpene excretion by the appendix of *Sauromatum guttatum*

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Communicated by Eugene W. Nester, University of Washington School of Medicine, Seattle, WA, July 12, 1995

ABSTRACT **Electron microscopy of the cells of the thermogenic appendix of *Sauromatum guttatum* has revealed a fusion event between pocket-like structures of the rough endoplasmic reticulum (rER) and the plasma membrane. As a result of the fusion event, many regions of the plasma membrane have paired unit membranes (four leaflets instead of two). The fusion allows the transfer of osmiophilic material from the rER pockets to the plasma membrane, where the osmiophilic material is confined to bilayer, pocket-like structures. A clear correlation is found between the presence of the osmiophilic compound and sesquiterpenes. Prior to heat production, the rER- and plasma-membrane pockets are electron dense, and sesquiterpenes are detectable only in tissue extracts. On the day of heat production, electron-translucent pockets are subsequently found and the stored sesquiterpenes are released to the atmosphere. Three sesquiterpenes have been identified by gas chromatography–mass spectrometry as α-copaene and β- and α-caryophyllene.**

Floral scent glands, osmophores, emit a wide array of odoriferous volatile compounds that are attractive to the flower's pollinators (1, 2). Most of the volatiles released by floral glands are lipophilic in nature, and many of them are terpenes (1). Five other types of secretory tissue in nonfloral organs secrete terpenes (3–5): (*i*) glandular trichomes (6–9); (*ii*) cavities circumscribed by specialized secretory cells (10, 11); (*iii*) resin ducts that are similar to secretory cavities, but more elongated (12, 13); (*iv*) laticifers, derived from an elongated cell or interconnected cells (14); and (*v*) secretory idioblasts, which are large cells (15). In many of these secretory structures the volatiles are either released upon synthesis (e.g., glandular trichomes) or stored in intercellular cavities (e.g., resin ducts).

Most floral glands are localized in small defined areas of the reproductive structures, or on the tips of petals and sepals (1). In the conspicuously long inflorescences of arum lilies (Araceae; not to be confused with true lilies, Liliaceae) the floral glands are situated along their spadix; in many species the glands form its specialized part, the naked appendix. The excretory pathway of their floral volatiles has never been studied adequately, and only one electron microscopy study, conducted almost 20 years ago on the appendix tissue of *Typhonium divaricatum* (another arum lily), makes mention of the presence of oily droplets near the plasma membrane (PM) (16, 17).

We report here on a route of excretion of terpenes from the interior of the floral gland cells of the *Sauromatum guttatum* appendix to the atmosphere. The appendix releases a variety of volatiles during its thermogenic activity. One group of volatiles is the sesquiterpenes that are accumulated and stored in the glandular cells before their release. The sesquiterpenes accumulate in rough endoplasmic reticulum (rER) pockets that fuse with the PM. The rER pockets are incorporated in the PM, and when the appendix becomes thermogenic the sesquiterpenes are released into the atmosphere together with other volatiles.

MATERIALS AND METHODS

Plant Material. Inflorescences of *Sauromatum guttatum* were allowed to develop in a growth chamber under a long-day condition (18). The developmental stage of the appendix was determined retroactively with respect to the day of inflorescence opening and heat production by the appendix (D-day; ref. 18).

Instrumentation. GC–MS analysis of the volatiles was performed on a VG MD 800 quadrupole mass spectrometer (VG BioTech Instruments, Cheshire, U.K.) directly interfaced to a Carlo Erba (model 8000) gas chromatograph equipped with a capillary split–splitless and on-column injector (Fisons Instruments, Manchester, U.K.). All analyses were carried out with a DB-1 fused-silica capillary gas chromatograph column [30 m × 0.32 mm (i.d.), 0.25-μm film thickness; J & W Scientific, Rancho Cordova, CA] operated with helium as carrier gas (head pressure, 10 psi). Headspace gases were analyzed in the splitless mode (injector temperature, 220°C) and "cold-trapped" on the column at 35°C. After 5 min, injector purge was initiated and the column oven temperature was programmed to increase linearly to 120°C at a rate of 4°C/min, then rapidly to 260°C, and held for 5 min. On-column analyses were carried out with the same temperature program. The mass spectrometer was operated in the electron ionization mode with a filament emission current of 100 μA and electron energy of 70 eV. The ion source and GC–MS interface temperatures were 200°C and 250°C, respectively. Data acquisition was in the scan mode (35–450 Da/0.5 sec) with mass calibration and daily tuning of the quadrupole by a 486DX50 personal computer and VG LABBASE software.

Cryogenic Trapping of D-Day Appendix Volatiles. The appendix, a slender 25-cm-long organ, still attached to the inflorescence was inserted into a port of an inverted 5-liter flask, whereas the other floral organs (male and female flowers and the club-shaped organs; ref. 18) remained outside the flask. The exit port of the flask was connected to a U-shaped glass tube filled with about 0.1 ml of hexane and inserted in liquid nitrogen. Air in the enclosed system was circulated at 30 ml/min. One microliter of the sample was subjected to GC–MS analysis. The volatiles were collected between 10:00 and 12:00.

Headspace Analysis of Appendix Volatiles. Portions (10 g) of appendix tissue were ground with a pestle and mortar and

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Abbreviations: ER, endoplasmic reticulum; rER, rough ER; PM, plasma membrane; D-day, day of inflorescence opening or of heat production; D–1, D–2, etc., days before D-day; D+1, one day after D-day.

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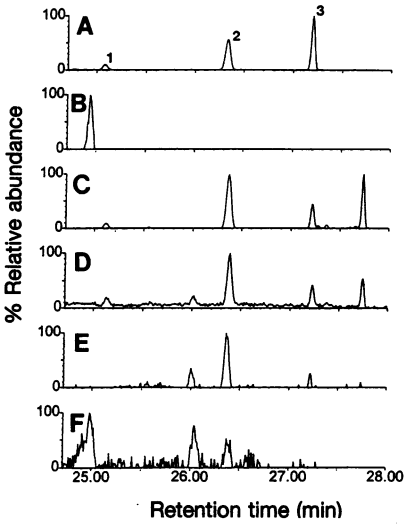

FIG. 1. Total ion chromatographs of the stored and released volatiles of the *Sauromatum* appendix. (*A*) Standards. (*B*) D−1 volatiles; the observed peak is a column artifact. (*C*) D-day volatiles. (*D*) D−1 extract. (*E*) D-day extract. (*F*) D+1 extract. *B* and *C* show cryotrapped volatiles in hexane. *D–F* show headspace analyses. Peak assignments: 1, α-copaene; 2, (−)-*trans*-caryophyllene (β-caryophyhllene); 3, α-humulene (α-caryophyllene). The ratio of total peak areas for *D*, *E*, and *F* is 1:0.003:0.02.

immediately placed in 10-ml headspace vials. After 2 hr at 35°C, 200 μl of headspace gas of each appendix was subjected to GC–MS analysis.

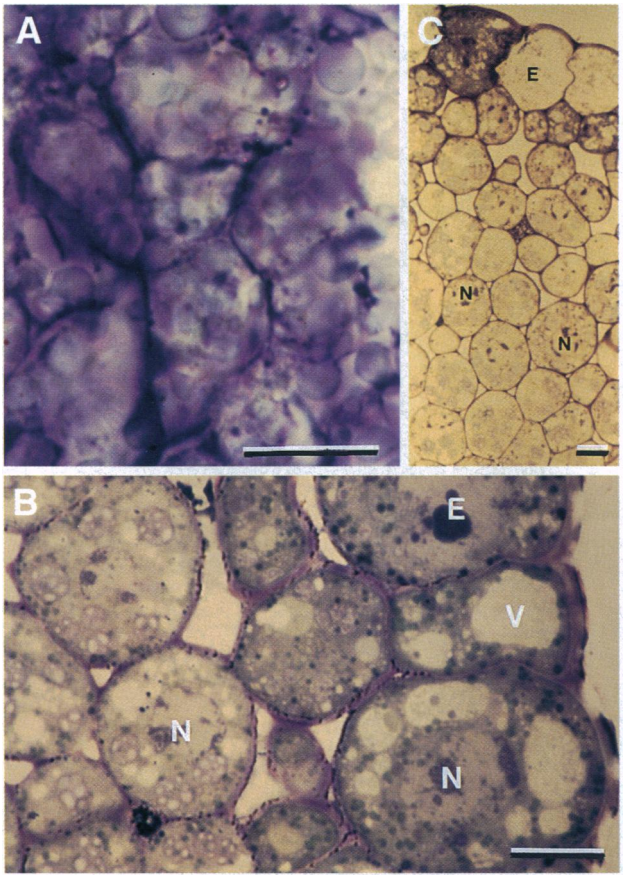

FIG. 2. Cells of the appendix of *S. guttatum* on D−2 (*A* and *B*) and D-day (*C*). (*A*) Unfixed tissue with dark-stained material localized mainly at the periphery of the cells. (*B*) Fixed tissue with dark-stained material at the periphery of and inside the cells. (*C*) Fixed tissue with no sign of dark staining. E, epidermal cell, N, nucleus; V, vacuole. (Bars = 4 μm.)

Analysis of GC–MS Data. The compounds corresponding to the various peaks were identified by comparison of their retention time and mass spectra with those of authentic standards purchased from ICN and with the National Institutes of Health/Environmental Protection Agency database.

Light and Electron Microscopy. Tissue blocks of a fresh D−2 appendix were frozen in liquid nitrogen. Cross sections of the blocks were cut (8 μm) on a cryostat (Leitz), stained with 0.1% toluidine blue O, washed, and examined under a light microscope in the absence of filters. Other tissue blocks were fixed in glutaraldehyde followed by osmium tetroxide (19). Ultrathin sections (silver to light-yellow interference color) from the subepidermal tissue (1–20 cell layers beneath the epidermis) were examined on a Philips model 410 electron microscope.

RESULTS

On D-day, the day of heat production, the *Sauromatum* appendix emits a mixture of volatiles compounds; among them are three sesquiterpenes: α-copaene and β- and α-caryophyllene (Fig. 1*C*). The sesquiterpenes are present in the tissue prior to D-day (Fig. 1*D*) but are not released (Fig. 1*B*). In the morning of the thermogenic activity, the levels of the sesquiterpenes are relatively high (Fig. 1 *C* and *E*), and on the next day, their relative levels dramatically decrease (Fig. 1*F*). Since extracts of immature appendices (1–3 days before heat production) release the same sesquiterpenes, we suggest that the

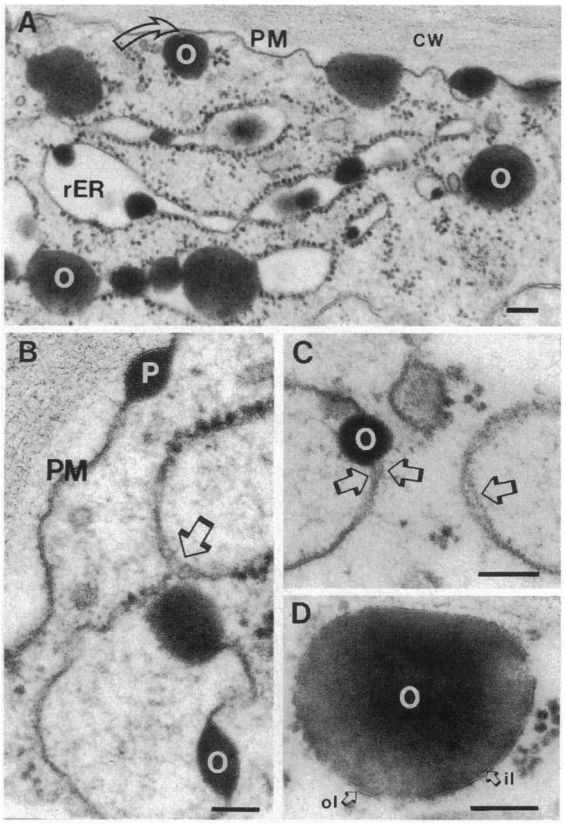

FIG. 3. Osmiophilic electron-dense material within the ER cisternae of a D−2 appendix. (*A*) rER is filled with osmiophilic material (O); curved arrow points to the membrane around that material. cw, Cell wall. (*B* and *C*) Paired unit membranes forming the pockets (P) in which the osmiophilic material accumulates; open arrows point to regions in the ER where two unit membranes are visible without osmiophilic material. (*D*) Osmiophilic material with a clearly visible surrounding membrane (ol, outer leaflet; il, inner leaflet). (Bars = 0.125 μm.)

sesquiterpenes are stored in the tissue before the thermogenic activity and are released during its activity.

GC–MS analysis of cryotrapped D-day-volatiles shows that monoterpenes are also released during the thermogenic activity (data not shown). However, they were not detectable in the headspace gas samples obtained from immature and D-day appendices.

When fresh unfixed appendix cells are stained with toluidine blue, a dark-blue color is developed in the periphery of the cells in contrast to the reddish cell walls (Fig. 2*A*). The developed color is atypical of stained lignified (blue-green) and unlignified cell walls (red) or of stained phenols (green; ref. 20). When sections are fixed and stained with osmium tetroxide and then with toluidine blue, there is a slight shift in the color developed on the periphery of the cells because osmium tetroxide intensely stains the sections black (Fig. 2*B*). This dark-stained material disappears on the day of heat and odor production (Fig. 2*C*).

Close electron microscopic examination of the appendix tissue shows that electron-dense, osmiophilic material is present only inside the cells and not in intracellular spaces. This material appears in the rER membrane (Fig. 3*A*). The rER has

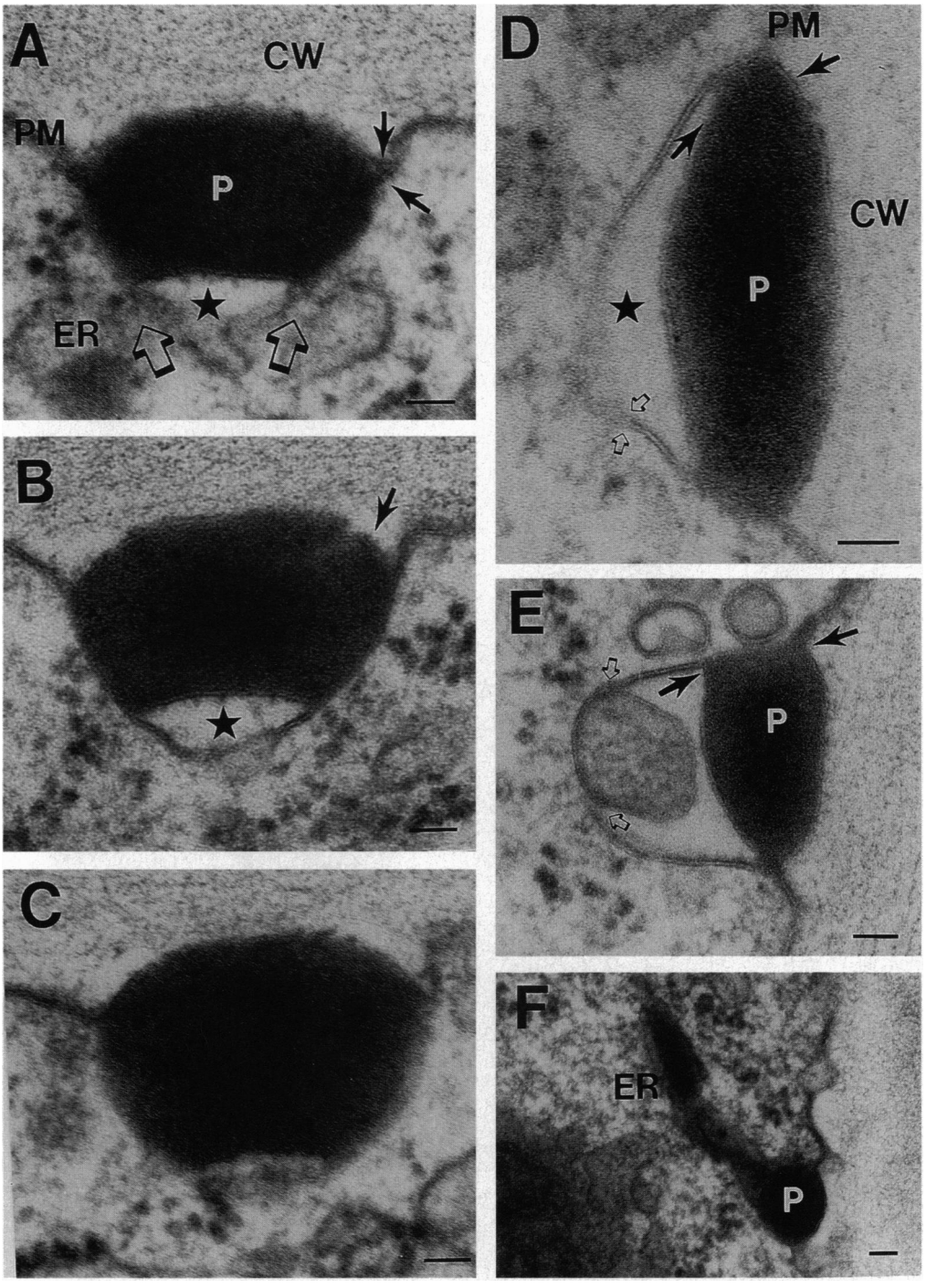

FIG. 4. Fusion between the ER and the PM. (*A–C*) Serial sections of a fusion site between the ER with a pocket-like structure (P) and the PM of a D−2 appendix. In *A* and *B* the lumen of the fused ER cisternae (star) is clearly seen, whereas in *C* it is disappearing. (*D*) High magnification of an ER pocket (P) fused with the PM but still connected to the ER membrane. (*E*) Another fusion site between the PM and the ER with a full (P) and an empty pocket. (*F*) on D-day morning, the pockets are still fused with the PM. Solid arrows point to the paired unit membranes of the pocket (P) and, open arrows point to the ER membrane. (Bars = 0.05 μm.)

membranous pockets in which the osmiophilic material accumulates (Fig. 3 *B* and *C*). The ER pockets are unbroken and ribosomes are attached to the ER membranes, suggesting that the osmiophilic material is not a result of dissolution of ER membranes or of the precipitation of ER substances. The ER bilayer membrane around the osmiophilic material is uniformly dense without any detectable precipitates, and the material is not found in the space between the two leaflets of the PM (Fig. 3*D*). This osmiophilic material conceivably represents compounds that accumulate and may even be synthesized in the ER.

The presence of material with identical osmiophilic density in the ER and the PM (Fig. 4) raises the possibility that the electron-dense material moves from the ER to the PM. The images in Fig. 4 *A–F* provide evidence for the existence of a route leading from rER pockets to the PM. Fig. 4 *A–C* show serial sections through an ER pocket fusing with the PM. In *A* the ER lumen is clearly evident, whereas in *C* it is retreating. *D* and *E* also show ER pockets incorporated in the PM while the ER membranes are still connected. On D-day morning, the ER pocket is still within the PM (Fig. 4*F*).

The ER pocket is fully integrated into the PM (Fig. 5 *A–E*). The contact sites with the PM are clearly visible, and the upper and lower membranes of these pockets are intact without any distortion or breakage. Some regions of the PM appear as four electron-dense lines (Fig. 5*F*) that could conceivably be created also by fusion between the ER and the PM (Fig. 5 *G* and *H*); the presence of empty ER pockets can be seen in Fig. 3 *B* and *C* and Fig. 4*E*. On D-day, the day that the appendix develops its spectacular heat and odor, the osmiophilic material has disappeared, and only electron-translucent membranous pockets are visible (Fig. 5*I*). From these images we conclude that the osmiophilic material most likely represents the appendix volatiles that are stored in the tissue before the

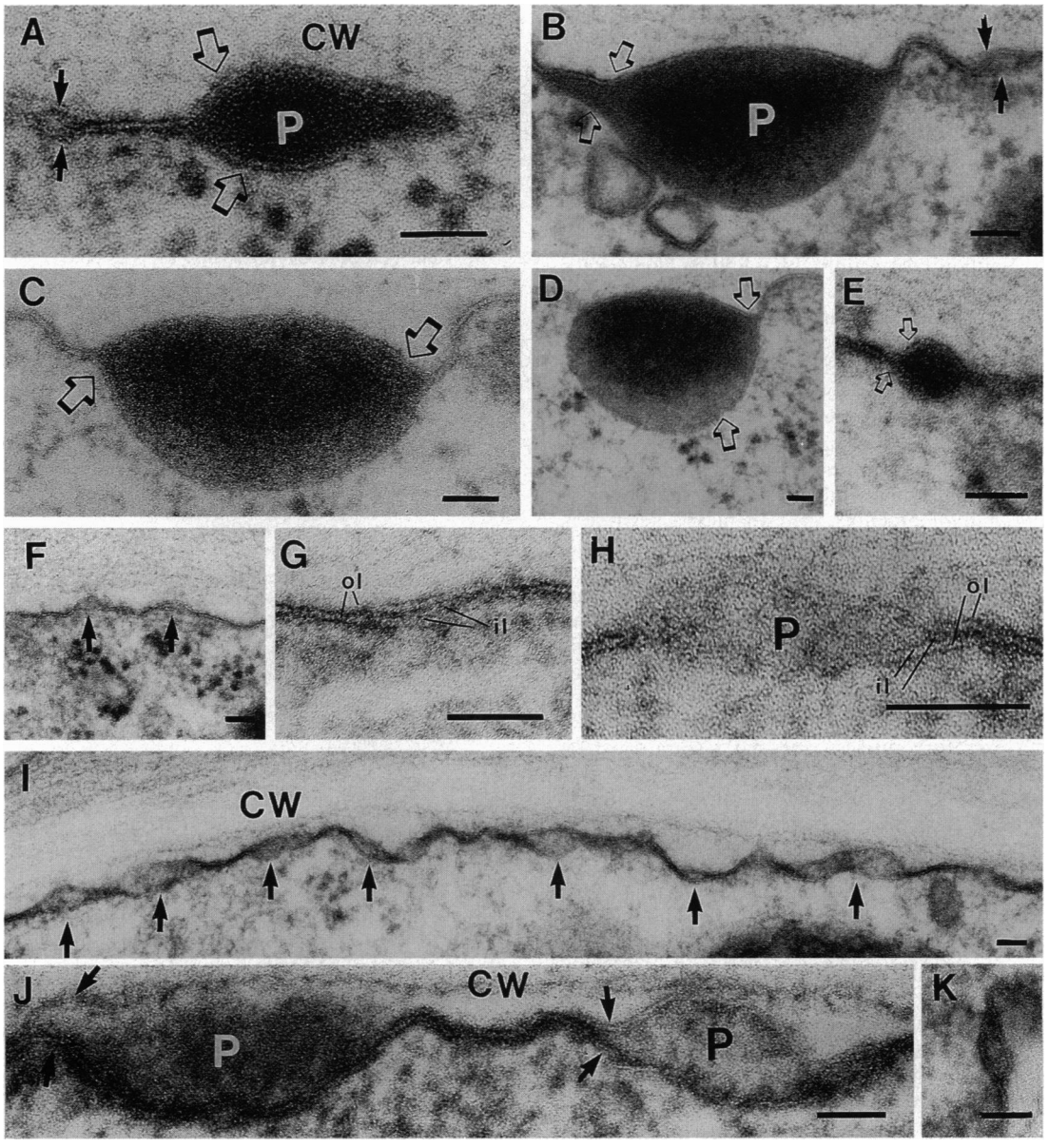

FIG. 5. The PM of the appendix cells examined 2 days before (*A–H*) and during (*I–K*) heat production. (*A–E*) Osmiophilic material accumulates in the membranous pocket (open arrows point to the paired "membrane unit" within which lipophilic material accumulates; solid arrows point to electron-translucent membranous pockets. (*F–H*) regions of the membrane with paired unit membranes—i.e., four leaflets instead of two. (ol, outer leaflets; il, inner leaflets). (*I*) Electron-translucent pocket-like structures devoid of osmiophilic material during heat production. (*J*) Two membranous pockets; one empty (on the right) and the second one partially empty (solid arrows point to the outer and inner membranes). (*K*) A small membranous pocket with clear contact sites between the paired membranes of the pocket structure and the PM. CW, cell wall; P, pocket-like structure. (Bars = 0.05 μm.)

thermogenic activity and are liberated during that activity. The best candidates to be represented by the osmiophilic material are the sesquiterpenes that are stored in the tissue and released on D-day. The presence of empty pockets suggests that the sesquiterpenes are released directly from these pockets to the exterior of the cells.

DISCUSSION

We have combined ultrastructural and chemical investigations to study the traffic of floral volatiles. We used the long, slender appendix of the *S. guttatum* inflorescence, which is composed of glandular tissue, as an experimental model. The secretory cells are not confined to a specific area of the appendix and are detected even at 20 cell layers beneath the epidermis.

From the electron microscopy data and the chemical analysis of the sesquiterpenes we conclude that the electron-dense material represents mainly three sesquiterpenes stored in the *Sauromatum* appendix: α-copaene, β-caryophyllene, and α-caryophellene. These sesquiterpenes occur widely in plants and are the result of different cyclizations (21). Further supportive facts are the chemical nature of sesquiterpenes and the absence of other likely candidates such as tannins (phenolic compounds). Osmium tetroxide can react instantly with the double bonds of sesquiterpenes and form precipitates similar to those seen in the electron micrographs. The precipitation of the sesquiterpenes inside cellular organelles provides information on their route from the interior to the exterior of the secretory cells. Neither the staining with toluidine blue nor the volatile analysis shows the presence of phenolic compounds, which also can be stained dark in the presence of osmium tetroxide.

The secretory process of sesquiterpenes in the appendix tissue is different from the exocytotic process, the most common fusion event in nonspecialized (22–24) and specialized (25, 26) cells, and in mammalian cells as well. In the exocytotic event, Golgi-derived vesicles fuse with the PM and their contents are deposited outside the PM. In the glandular cells of the *Sauromatum* appendix, the rER plays a key role in the biosynthesis, accumulation, and secretion of sesquiterpenes. The rER pockets fuse with the PM and their contents are not deposited outside the PM but stay inside the pockets, which become fully incorporated into the PM. Since these compounds are toxic, they are surrounded by a membrane to avoid deleterious effects on cellular functions and are released only during the thermogenic activity.

Many mitochondria are present in these cells to provide the energy for the excretory process and the respiratory heat needed to facilitate the evaporation of the volatiles. It is conceivable that the heat generated by the mitochondria causes an increase in the vapor pressure of the sesquiterpenes, and consequently their evaporation from the pockets to the exterior of the cells is facilitated. Another intriguing explanation is that changes in membrane properties on D-day allow the diffusion of sesquiterpenes from the PM pockets to the exterior of the cells. In a recent electron microscopy study, we observed changes in the ultrastructure of many components of the appendix cells during heat production (19).

Our findings contrast with previous studies (3, 8, 9) on excretion of lipophilic volatiles that have suggested that cytoplasmic lipophilic material diffuses through the PM of the epidermal cells or the trichome cell in the form of single molecules which then aggregate into droplets outside the epidermal cells (3) or, alternatively, that the cytoplasmic droplets are internalized by the PM and then released to the outside of the cell (3, 8, 9).

Other members of the Araceae produce heat and aroma during anthesis, and it is likely that the volatile compounds are released in a similar mechanism. Plants of other families (Asclepiadeae, Aristolochiaceae, Orchidaceae, and Burmanniaceae) have osmophores that release terpenes as pollinator attractants (1, 27, 28). We are confident that our findings can be generalized to include other arum lily species, such as *T. divaricatum*, and even the flowers belonging to the other families.

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