Int. J. Plant Sci. 154(1):107–118. 1993.

DEVELOPMENT AND FINE STRUCTURE OF THE GLANDULAR TRICHOMES OF ARTEMISIA ANNUA L.

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Development of capitate glands on the leaves of annual wormwood (*Artemisia annua* L.) was monitored with scanning and transmission electron microscopy. Differentiation of foliar cells into gland cells began in the youngest leaf primordia. After differentiation into a 10-celled biseriate structure of two stalk cells, two basal cells, and three pairs of secretory cells, the cuticle of the six secretory cells separated from the cell walls to form a bilobed sac that eventually splits to release its contents. At every developmental stage, the cells of the the gland contained relatively little vacuolar volume. The secretory cells contained extensive endoplasmic reticulum. The plastids of each cell pair were different. At maturity, the apical cells contained proplastids or leucoplasts with only occasional thylakoids. The cell pair below the apical cell pair contained amorphous chloroplasts without starch grains. The basal cell pair contained proplastids or leucoplasts and the stalk cells contained chloroplasts. The stroma to thylakoid ratio in the secretory cell chloroplasts was high. Initially, osmiophilic product was observed most freqently associated with stacked thylakoids, plastid envelopes, and smooth endoplasmic reticulum, although it was associated with all cell membranes. Near the plasma membrane adjacent to cell walls bordering the subcuticular space, the cytoplasm was enriched in smooth endoplasmic reticulum containing osmiophilic material. The apical cell wall of the apical secretory cell pair was reticulated on the inner cytoplasmic side and contained osmiophilic staining on the cuticular side. During early senescence, osmiophilic product was commonly associated with outer mitochondrial membranes.

Introduction

The glandular trichomes of the genus *Artemisia* are the site of accumulation of a variety of secondary metabolic products (Slone and Kelsey 1985). *Artemisia* species are the source of many sesquiterpenoid lactones (Duke et al. 1988; Marco and Barbera 1990), and some of these have been histochemically located in the subcuticular space of the head cells of the glandular trichomes of *Artemisia umbelliformis* Lam. (Cappelletti et al. 1986). The antimalarial drug artemisinin, a sesquiterpenoid lactone, is produced by *Artemisia annua* (annual wormwood; Klayman 1985). The leaves and stems of this species are covered with glandular trichomes (Duke et al. 1988).

There is considerable worldwide interest in the production of this valuable compound (Klayman 1985; World Health Organization 1988; Bryson and Croom 1991), yet little is known of the development and structure of the glands in which it is assumed to accumulate. There is also interest in this plant, as a dried herb for the floral and craft trades, where it is used in aromatic wreaths (Simon et al. 1990), and as a potential source of naturally occurring pesticides (Duke et al. 1987, 1988; Chen and Leather 1990; Chen et al. 1991; DiTomaso and Duke 1991; Duke 1991). Many of the compounds found in *A. annua* (Charles et al. 1991) are bioactive and phytotoxic (Duke et al. 1988; Duke 1991). Artemisinin is highly phytotoxic to *A. annua* itself (Duke et al. 1987), making sequestration within or secretion from the producing tissues a necessity. Autoallelopathic compounds of velvetleaf (*Abutilon theophrasti*), are found only in glandular trichomes (Sterling et al. 1987). The subcuticular spaces of the glandular trichomes are likely sites of sequestration of artemisinin and other phytotoxic secondary compounds produced by *A. annua*. Nothing has been published on the structure and development of the glands of this species.

In this article we examine the developmental structure of the specialized cells of *A. annua* glandular trichomes at the scanning and transmission electron microscope levels. Special emphasis has been placed on the plastids of these cells because the plastids have distinctive ultrastructural and morphological features. The plastids of the glandular trichome secretory cells were found to be specialized and structurally dissimilar to plastids in other parts of the plant. Others have previously noted that plastids of resin-secreting cells sometimes have plastids with unusual morphology (Dell and McComb 1978*b*).

Material and methods

Plant material

Seeds of *Artemisia annua* were planted shallowly (1–2 mm) in 1.2-L pots filled with Jiffy-Mix (Ball Jiffy) potting media. The pots were watered with tap water once every 4 d until seedling establishment and then watered every other day with a dilute solution of Peters 20-20-20 general purpose fertilizer (0.25 g/L). Leaves from mature plants 30–60 d old were sampled for microscopy studies.

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Manuscript received June 1992; revised manuscript received October 1992.

Scanning electron microscopy (SEM)

Meristematic and mature leaf tissue was fixed overnight in 4% glutaraldehyde in 0.1 M cacodylate buffer. After washing in distilled water and dehydrating in ethanol, the tissues were dried in a critical-point drier (Balzers 020). The dried specimens were then mounted on aluminum specimen stubs with silver conductive paint, coated with 20 nm of gold-palladium in a sputter coater (Anatech Hummer X), and observed and photographed in a scanning electron microscope (JEOL JSM-840) at 15 kV. In addition, SEM was performed on the etched faces of resin-embedded material prepared as described below. Etching was accomplished by immersing the faced specimen block for 5 min in a saturated solution of sodium hydroxide in absolute ethanol (sodium ethoxide).

Transmission electron microscopy (TEM)

Meristematic and mature leaf tissue was fixed for 4 h in 0.2% glutaraldehyde in 0.1 M cacodylate buffer. After rinsing for 30 min in the buffer, the tissue was postfixed for 2 h in 1% OsO_4 in 0.5 M cacodylate buffer. The tissue was then rinsed in distilled water, dehydrated in an acetone series, and embedded in Spurr's resin. Thin sections obtained with a diamond knife on an ultramicrotome (Reichert-Jung Ultracut E) were stained with 2% uranyl acetate for 5 min and poststained with Reynolds's (1963) lead citrate for 5 min. The sections were observed and photographed in a transmission electron microscope (Zeiss EM 10CR) at 60 kV.

Morphometric analysis

Cross-sectional area morphometry was performed using a digital planimeter (Lasico Model 40). The instrument was traced over chloroplasts in micrographs of known cell type and magnification. The resultant reading was used to calculate the cross-sectional area of each plastid within a cell, according to

$$CA = \left(\frac{1}{mag \times 0.0001}\right)^2 \times \mu \text{ metric},$$

where CA = area calibration constant; mag = micrograph magnification; μ metric = value of one planimeter unit (a constant for the instrument). The planimeter readout for each measurement was multiplied times CA to convert it to μm^2. Checks were done for each magnification by drawing a figure of known area and performing planimetry on it.

The internal volume densities of the components of chloroplast types were compared by the method of Mayhew and Reith (1988). Micrographs were printed with a superimposed grid, and the intersections of the lines were used for point counts. Each point overlaying a chloroplast was recorded according to the chloroplast constituent on which it fell. The number of hits per constituent was divided by the total chloroplast hits to obtain the volume density for the chloroplast component.

Results

Scanning electron microscopy

At apical meristems, SEM showed early stages of formation of capitate glands on leaf primordia (fig. 1*A*). Glands at various stages of early development densely covered the leaf primordia. The most mature glands had a biseriate configuration of 10 cells, five on each side (fig. 1*B*). At this stage, no filamentous trichomes were seen on the leaf surfaces. Later during the early leaf development the capitate glands were sometimes obscured by filamentous trichomes (fig. 1*C*). On the adaxial surfaces of young leaves, filamentous trichomes and capitate glands were arranged in two rows in troughs along either side of the leaf midrib (fig. 1*C*). The pattern of glands on the abaxial leaf surface and on stems was random (fig. 1*D*).

By these stages, the capitate glands had expanded, and the glands had taken on a characteristic bilobed shape of a mature structure resulting from the expansion of the cuticle covering the apical cells of the gland (fig. 2*A, B*). The cuticular sac was oroken in many of the more mature glands (fig. 2*C*). Fractured and etched preparations also revealed the internal cellular structure of mature gland cells (fig. 2*D*). The cellular contents of the gland cells (fig. 2*D*) were more dense and less vacuolated than those of the underlying mesophyll cells (not shown).

Transmission electron microscopy

The earliest stage of gland formation observed was the one-cell stage, in which a single epidermal cell enlarged to protrude above the leaf surface (fig. 3*A*). After considerable expansion above the plane of the leaf, this cell divided anticlinally (fig. 3*A, B*), and then both of the resulting cells divided periclinally (fig. 3*C*). In the one- to four-cell stage, vacuoles were relatively small and plastids were proplastids with only a few unstacked thylakoids.

The six-cell stage was formed by periclinal division of the apical gland cells (fig. 3*D*). All cells of the six-cell stage contained chloroplasts with few stacked thylakoids and no starch grains (fig. 3*D*). At this stage, lack of starch grains was the only distinguishing feature between gland chloroplasts and those of the mesophyll tissues.

The final 10-cell stage of the gland was the result of further periclinal cell division of the two

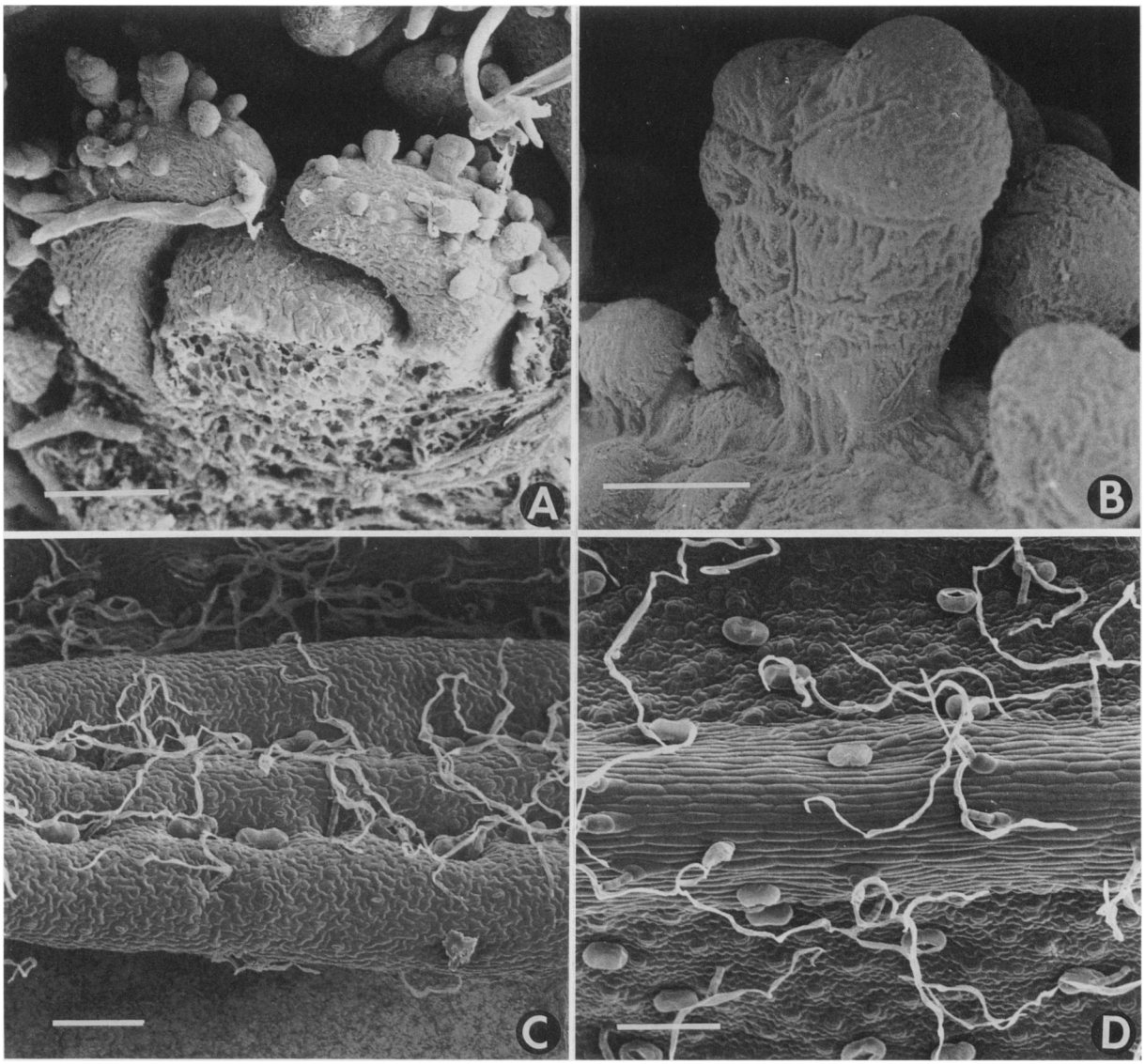

Fig. 1 SEM of various stages of capitate gland development of *Artemisia annua*. *A*, Apical meristem with leaf primordia covered with developing glands. Scale bar = 50 μm. *B*, Individual immature 10-cell gland before cuticular separation. Scale bar = 10 μm. *C*, Adaxial view of leaf with glands in which the cuticular sac has filled. Scale bar = 100 μm. *D*, Abaxial view of mature leaf showing glands on midrib. Scale bar = 100 μm.

apical cell layers (fig. 4). There were three apical cell pairs that we term secreting cells because, at later developmental stages, the subcuticular space was bordered by these cells. The subcuticular space is filled with secretory products that cause the cuticle to expand to its bilobed external appearance. Below these cells were two basal cells and a stalk cell pair, attaching the gland to the leaf epidermis. The gland cells (fig. 4) are more vacuolated than those of the average gland at this stage. The outer portion of the cuticle stained more intensely than the leaf cuticle at this stage. After all 10 cells had been formed, the cuticular surface of the gland began to separate from the cell wall near the tip of the gland (fig. 4, inset). The onset of cuticular detachment may be associated with the onset of secretory activity. Little or no osmiophilic material was found in the cells at this point; however, osmiophilic material could be seen below the cuticle, in the outer portion of the cell wall.

The plastids of the two apical cells at the 10-cell stage were amoeboid and contained no thylakoids, whereas those of the three cell pairs distal to the apical cell pair were amoeboid with thylakoids. Those of the basal cells contained occasional thylakoids. Plastids of the two stalk cells contained thylakoids and starch grains.

The detachment of the cuticle from the external cell walls of the gland cells continued down to the two stalk cells above the basal cells, resulting in a subcuticular space bordered by the six most

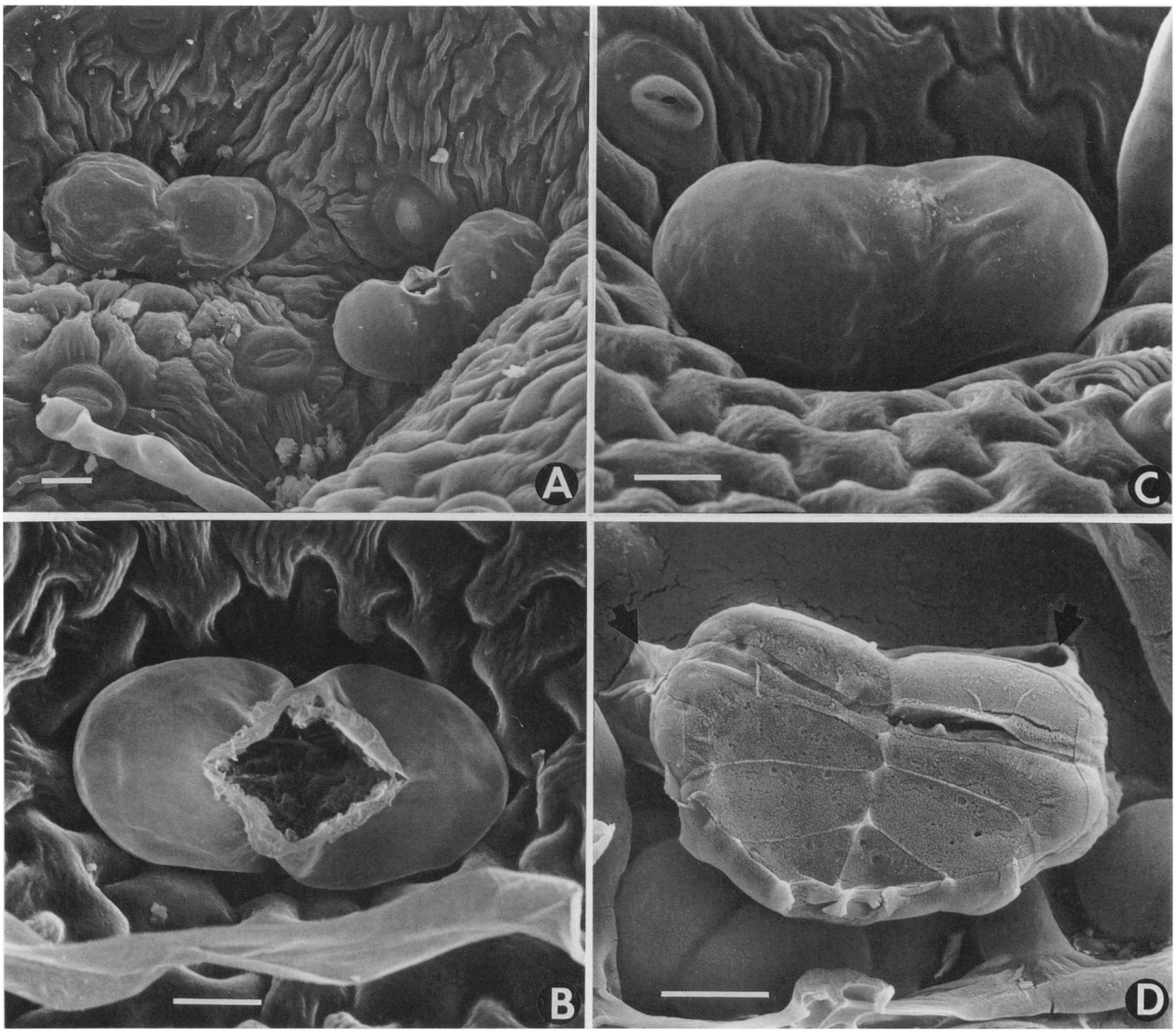

Fig. 2 SEM of mature capitate glands of *Artemisia annua*. *A*, Glands on typical leaf surface. *B*, Higher magnification of fully expanded gland. *C*, Mature gland after splitting of cuticular sac. *D*, Fractured and etched view of glandular cells. All gland cells and the detached cuticle (arrows) are resolved. Scale bars = 10 μm.

apical cells of the gland (fig. 5*A*). The cytoplasm was denser at this stage than earlier (compare with fig. 4). At this point, the two basal cells contained chloroplasts and relatively large vacuoles.

The apical cell pair generally had no chloroplasts, and their plastids resembled large proplastids. The cell walls of these two cells had reticulated ingrowths, as with transfer cells, that served to increase surface area, possibly to facilitate product export (fig. 5*B*). Osmiophilic material was often associated with the plasma membrane/cell wall interface of the reticulations, obscuring the plasma membrane. Cytoplasm near this interface included a high density of smooth endoplasmic reticulum (ER) containing osmiophilic material. The outer cell walls of the apical portions of the apical cell pair contained osmiophilic material with a granular appearance, perhaps from filling interlinked pores in the cell wall. Osmiophilic material was sometimes found between plasma membranes and cell walls that did not border the subcuticular space (fig. 5*C*). The cytoplasm near such plasma membranes contained a high density of osmiophilic smooth ER.

The subapical two cell pairs (four cells) contained large, amorphous chloroplasts without starch grains. The basal cell pair between the secretory and stalk cells contained leucoplasts with osmiophilic globules attached to their outer envelopes. The two stalk cells often contained smaller chloroplasts.

During this stage of development, we assume that the secretory glands are exporting relatively

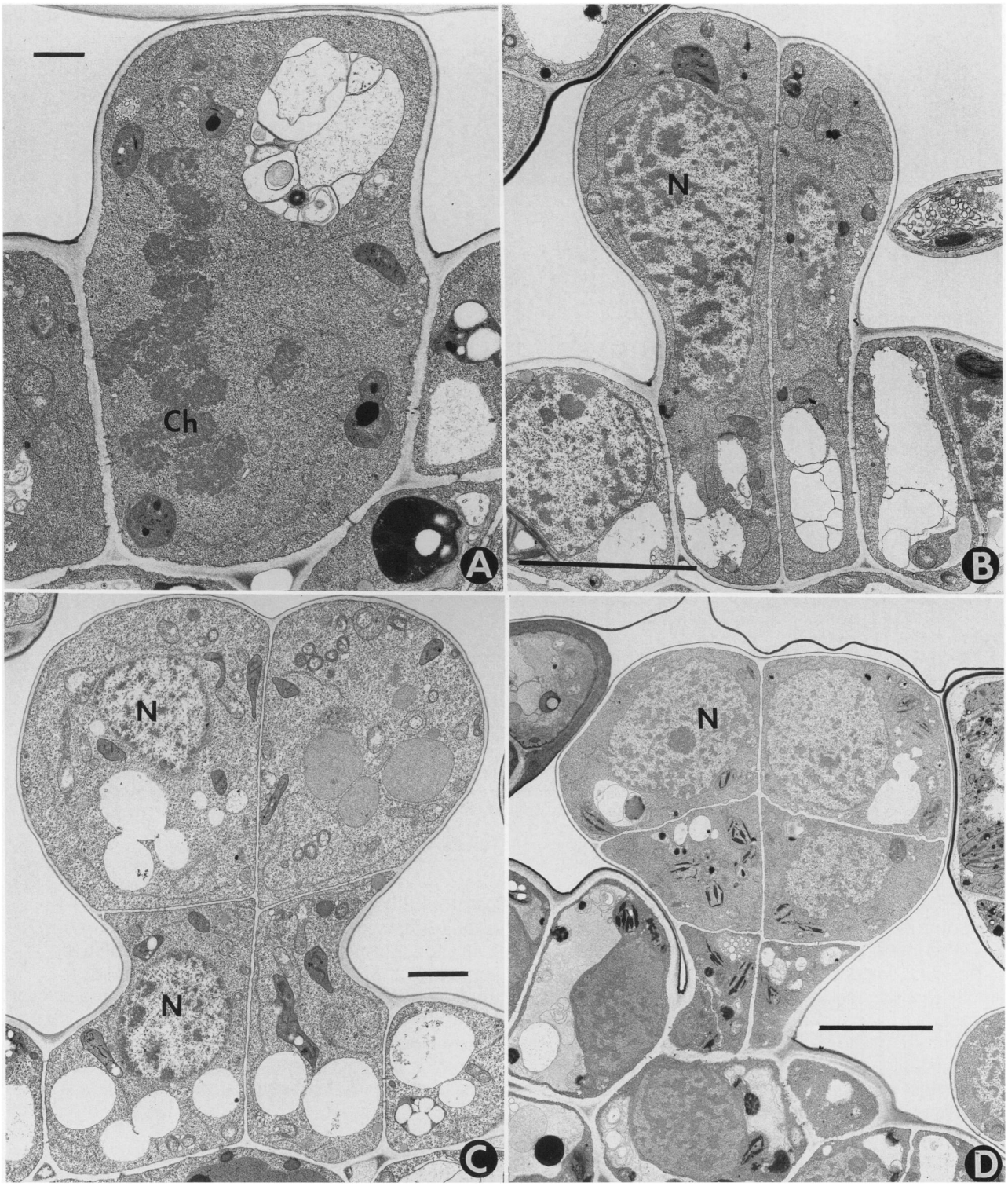

Fig. 3 TEM of early stage of development of capitate glands of *Artemisia annua*. *A*, One-cell stage. This cell is probably in early prophase of the first anticlinal division. *Ch* = condensed chromatin. Scale bar = 1 μm. *B*, Two-cell stage. *N* = nucleus. Bar = 5 μm. *C*, Four-cell stage following second (periclinal) division. Bar = 2 μm. *D*, Six-cell stage. Bar = 5 μm.

large amounts of product into the subcuticular space, as its expansion is rapid at this time. Most of the secretory material of the subcuticular space was apparently lost during fixation.

At this stage, much of the osmiophilic material first appeared associated with the envelope and thylakoids of plastids and the smooth ER (fig. 6). The chloroplast was the only organelle to contain

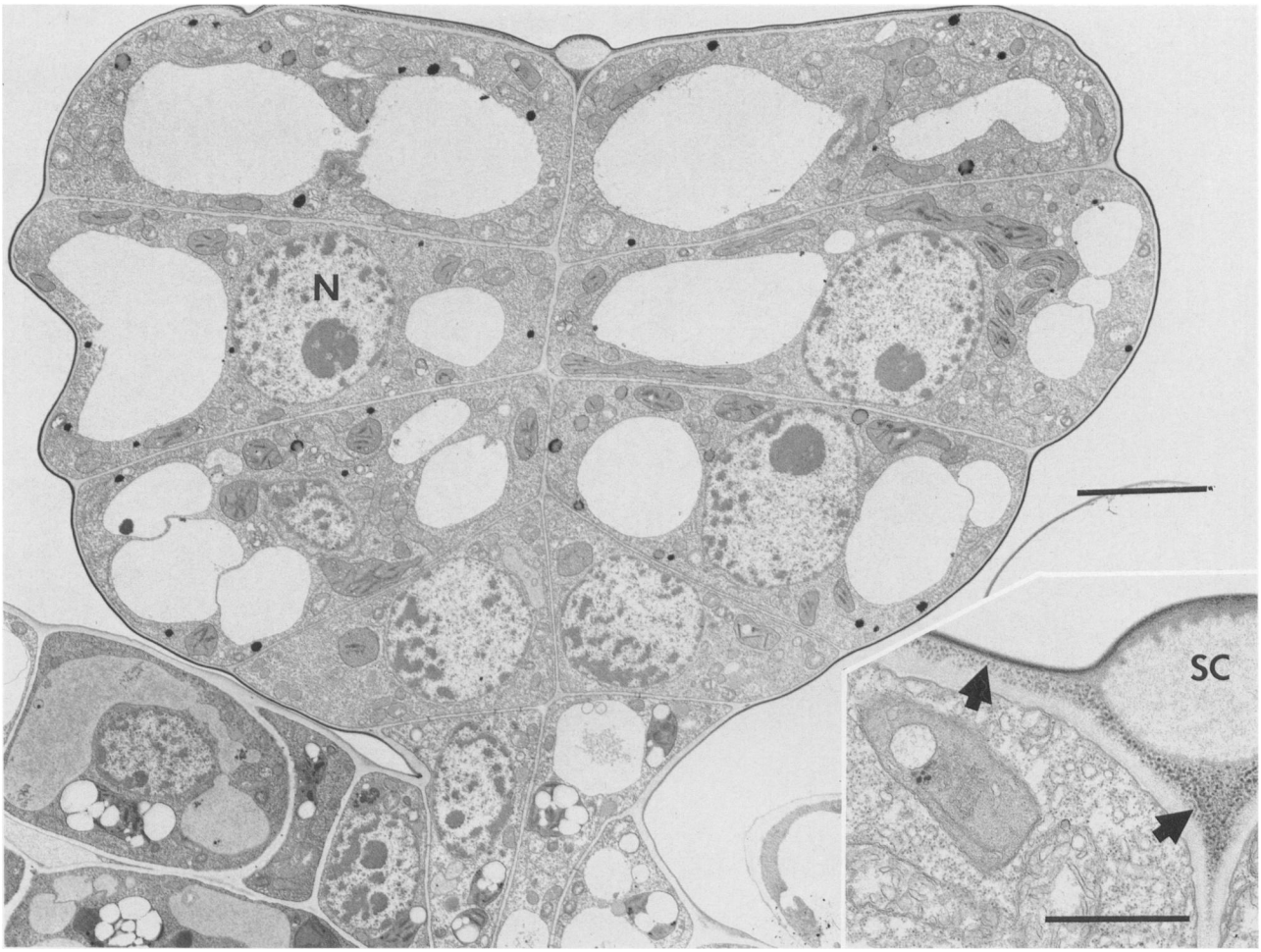

Fig. 4 TEM of 10-cell stage gland of *Artemisia annua* at the beginning of secretory cavity (cuticular sac) formation. The cuticle has begun to detach from the cell wall at the boundary of two apical cells. Bar = 5 μm. Inset at higher magnification shows accumulation of osmiophilic material (arrows) in the outer cell wall, below the the cuticle. *SC* = secretory cavity. Scale bar of inset = 1 μm.

large amounts of osmiophilic material. Large amounts of osmiophilic material were often observed between the plasmalemma and cell wall (fig. 6*A*). Mitochondria were abundant in these cells at this stage of development (fig. 6*A*). The plastids of the two apical cells contained less osmiophilic material than chloroplasts of the next two cell layers. Osmiophilic material could have been lost or redistributed during fixation. Chloroplasts often had only stacked thylakoids, without intergranal, nonstacked thylakoids. Grana in such chloroplasts were not aligned (fig. 6*B*). Some chloroplasts contained prolamellar body-like structures (not shown). Osmiophilic material of the gland plastids was relatively amorphous (fig. 6*A–C*), compared with the globular osmiophilic material of mesophyll plastids (fig. 6*D*). The thylakoids were intimately associated with this osmiophilic material, appearing sometimes to abut or envelop the material (fig. 6*A–C*).

As the glands matured further, the amount of osmiophilic material increased to the point that good ultrastructural definition was difficult to obtain, and normal plastid structure began to deteriorate (fig. 7). The osmiophilic material at this stage could in part result from membrane deterioration caused by senescence. At this stage, the plasmalemma appeared to be separated from the cell wall, perhaps because of deposition of material between the cell wall and the plasmalemma. The cell wall of the apical cells of the gland that bordered the subcuticular space had an osmiophilic layer of granular appearance that at the gland apex was thicker than the nonosmiophilic portion of the cell wall (fig. 7*B*). In this micrograph, the reticulated inner side of the cell wall can also be seen. Osmiophilic globules were more commonly associated with the outer membranes of mitochondria or contained within other intracellular membranes. No intercellular spaces developed between gland cells at any point during gland development. After this stage, cellular

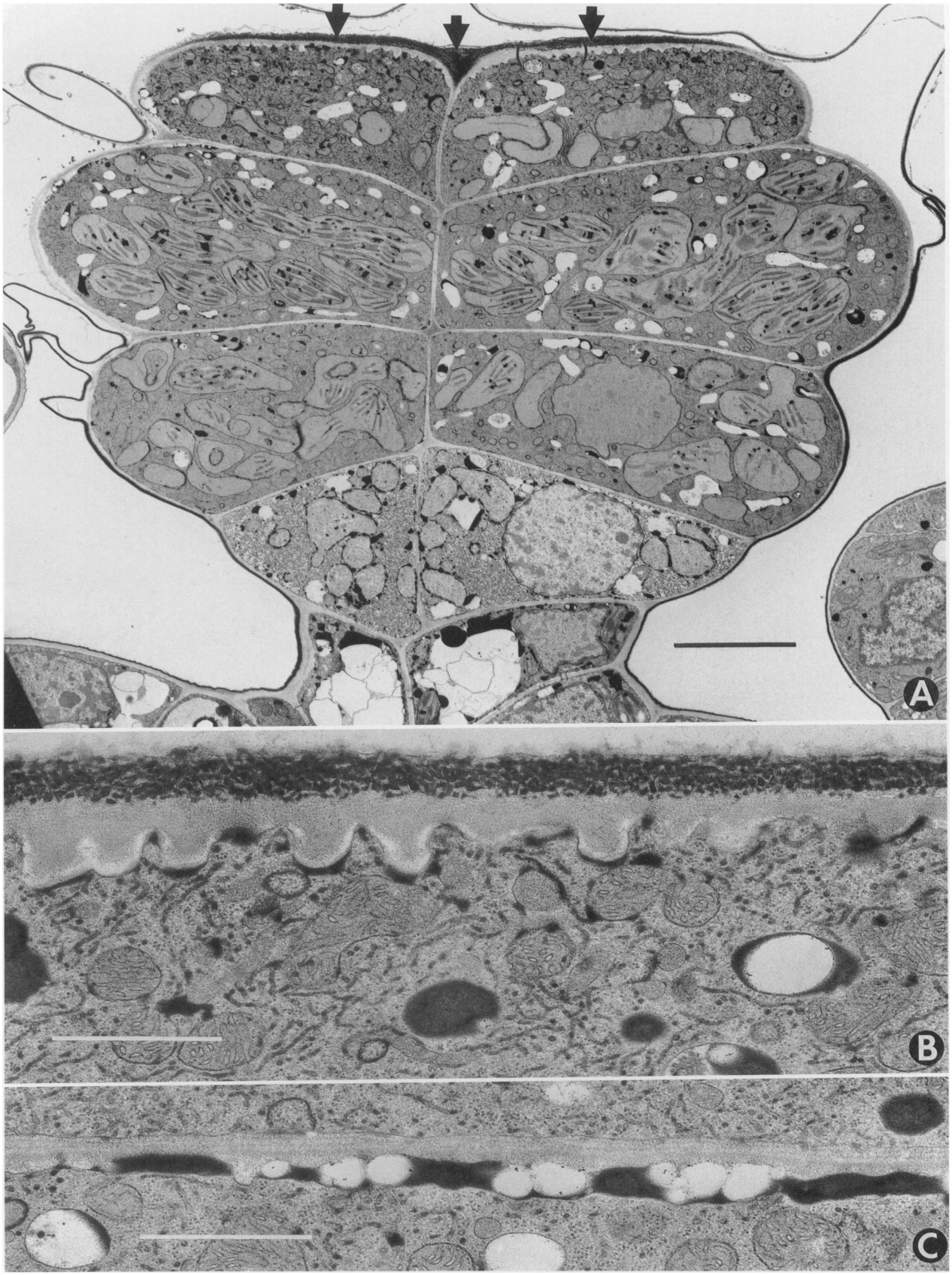

Fig. 5 TEM of 10-cell stage gland of *Artemisia annua* just after the cuticular expansion. *A*, A dense layer of osmiophilic material (arrows) comprises a portion of the cell walls of the two apical cells. Cell wall reticulations (parentheses) increase the surface area of the plasmalemma in this region. Scale bar = 5 μm. *B*, Higher magnification of reticulated cell wall and cytoplasm containing smooth endoplasmic reticula with osmiophilic material from an apical cell. Scale bar = 1 μm. *C*, Cell walls between apical and subapical cells. Scale bar = 1 μm.

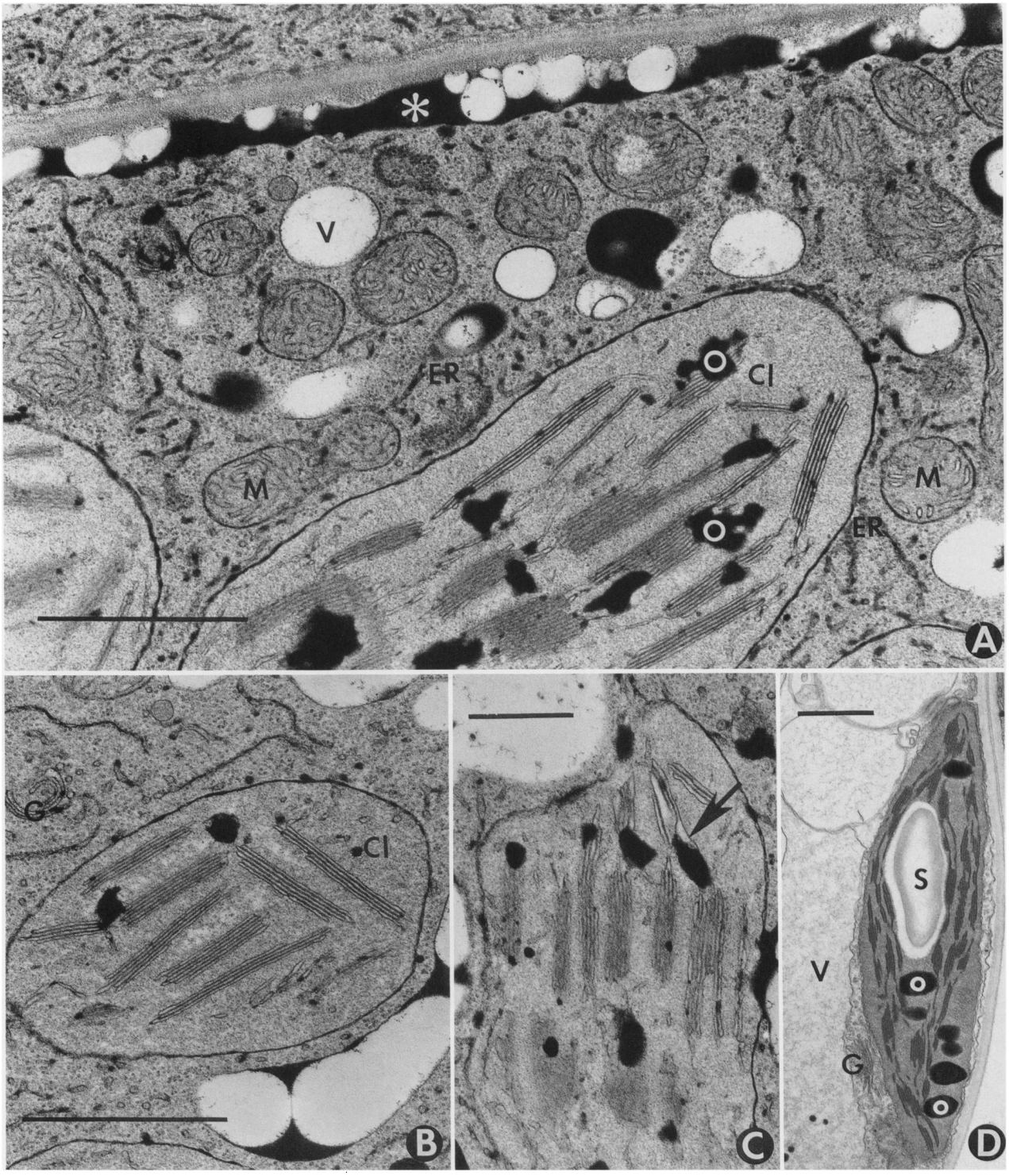

Fig. 6 TEM of plastids from subapical gland cells of *Artemisia annua* at the stage of figure 5. *A*, Micrograph of a chloroplast involved in product production. Osmiophilic material (*O*) seems to be produced at the edges of grana stacks. Note deposition of product between the cell wall and plasmalemma (asterisk). *Cl* = chloroplast; *ER* = endoplasmic reticulum; *M* = mitochondria; *V* = vacuole. *B*, Smaller plastid. Note lack of grana alignment. *G* = Golgi body. *C*, Plastid with large osmiophilic mass apparently between two thylakoid membranes (arrow). *D*, Mesophyll chloroplast with globular osmiophilic inclusions (*O*), starch grain (*S*), and associated Golgi body (*G*). Scale bars = 1 μm, except *C*, which is 0.5 μm.

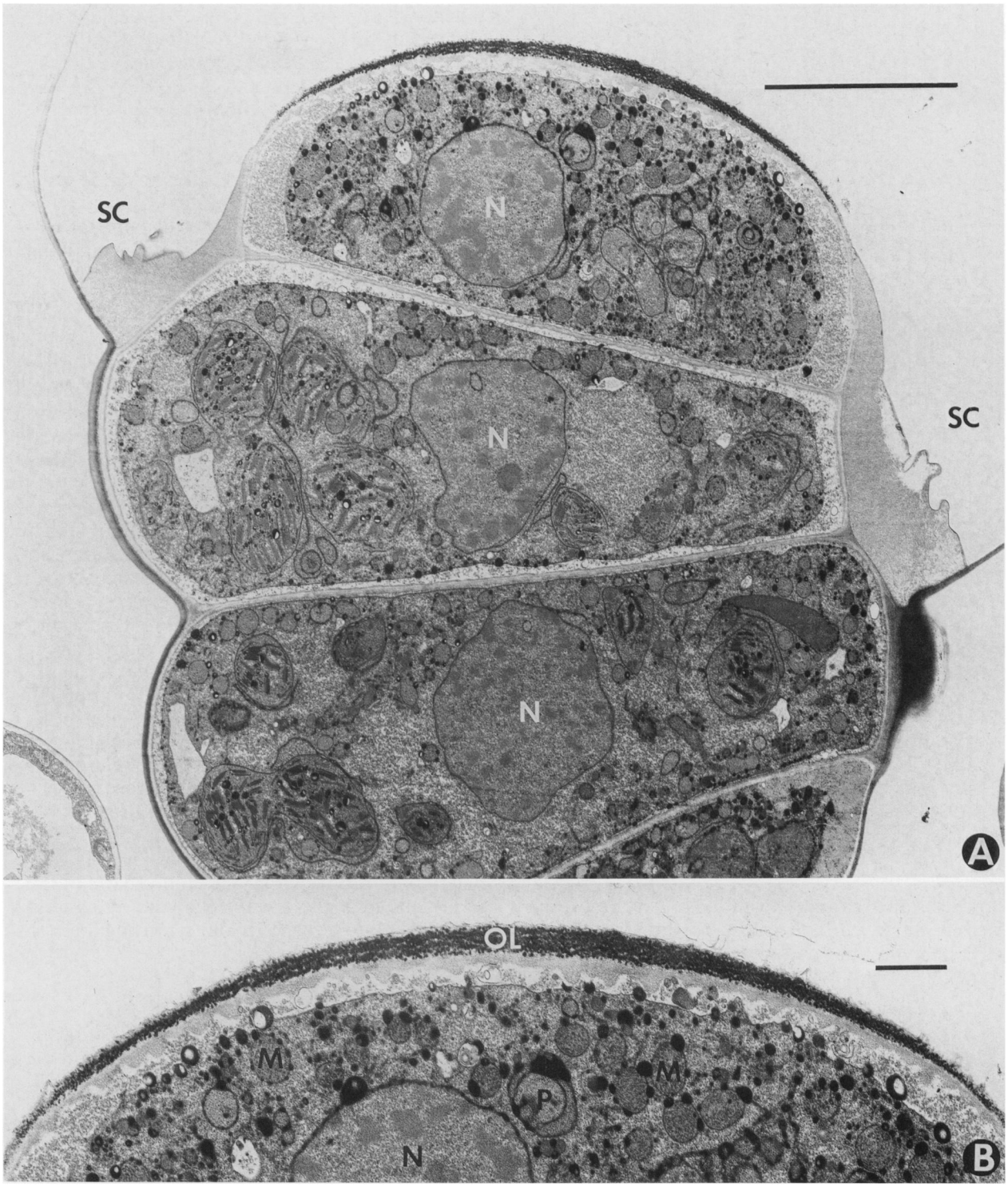

Fig. 7 TEM of gland cells of *Artemisia annua* at the beginning of gland senescence. Note the shrinkage of the plasma membrane from the cell wall and loss of organelle definition. *A,* Three cells from the three most apical cell layers. *N* = nucleus; *SC* = subcuticular space. Scale bar = 5 μm. *B,* Enlarged view of apical portion of apical cell. *M* = mitochondria; *P* = plastid; *OL* = osmiophilic layer. Scale bar = 1 μm.

membranes began to break, resulting in loss of organellar and cellular integrity (not shown).

Morphometric analysis of chloroplasts of the subapical two cell pairs of young, but mature, glands (fig. 5) showed that the average area of secretory cell chloroplasts of the subapical cell pairs was 6.1 μm^2, compared with 6.5 μm^2 for mesophyll chloroplasts of mature mesophyll tissues (not shown). Although the distribution of chloroplast sizes was similar between the two

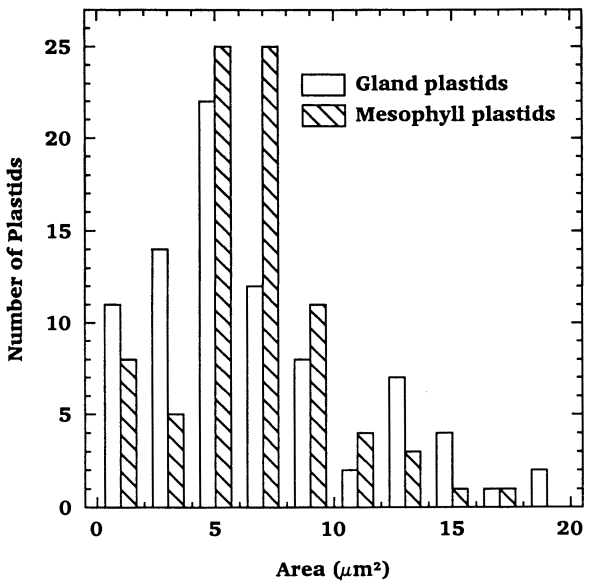

Fig. 8 Distribution of cross-sectional areas of plastids of cells of subapical mature cell pairs (see fig. 5) and those of mature mesophyll cells of *Artemisia annua*. The same number of plastids was counted of each cell type.

plastid types, the mesophyll plastids were more uniform in size (fig. 8) and shape. Mesophyll chloroplasts (fig. 6*D*) were typically ellipsoid with distensions caused by starch grains, whereas gland cell chloroplasts were isodiametric to amoeboid (figs. 4–6). The stroma to thylakoid volume density ratio within these gland chloroplasts was only slightly higher than in mature mesophyll cells, if the volume density of starch grains of the mesophyll chloroplasts was subtracted (fig. 9).

Discussion

Biseriate, capitate glandular trichomes are common in certain genera of the Compositae (Fahn 1988) such as *Artemisia* (Kelsey and Shafizadeh 1980; Ascensão and Pais 1982, 1985, 1987; Slone and Kelsey 1985; Cappelletti et al. 1986) and *Chrysanthemum* (Vermeer and Peterson 1979*a*, 1979*b*; Peterson and Vermeer 1984). The glandular trichomes of *Artemisia annua* that we have described are quite similar to those of *Artemisia umbelliformis* (Cappelletti et al. 1986). However, in *A. umbelliformis* the biseriate glandular trichomes were obscured on leaves by other types of trichomes. Histochemical stains for sesquiterpenoid hydroperoxides resulted in staining of the subcuticular space and head cells of the glandular trichomes. *Artemisia campestris* glandular trichomes (Ascensão and Pais 1985, 1987) were virtually identical to those of *A. annua* except that the amoeboid chloroplasts that we have described in the subapical two cell pairs were not noted in studies with *A. campestris*. Ascensão and Pais (1987) found chloroplasts only in the second and third cell pairs (from the base) in the glands of *A. campestris*.

Others have noted head or stalk cells of capitate glands containing chloroplasts without starch grains (Vermeer and Peterson 1979*b*; Ascensão and Pais 1982). The plastids of soybean (*Glycine max*) glandular trichome cells contain starch grains but have no thylakoids (Franceschi and Giaquinta 1983). In chloroplasts, starch accumulation indicates that production exceeds export. In the tissues that we sampled, mesophyll chloroplasts contained high levels of starch (fig. 6*D*). Thus, if photosynthetic rates of gland and mesophyll chloroplasts were similar, it follows that the glandular chloroplasts are exporting photosynthate at a dramatically higher rate than mesophyll chloroplasts, or starch metabolism differs between the two chloroplast types. This is consistent with the view that the chloroplasts of the gland cells are highly involved in converting photosynthate into terpenoids and exporting them to the cytoplasm.

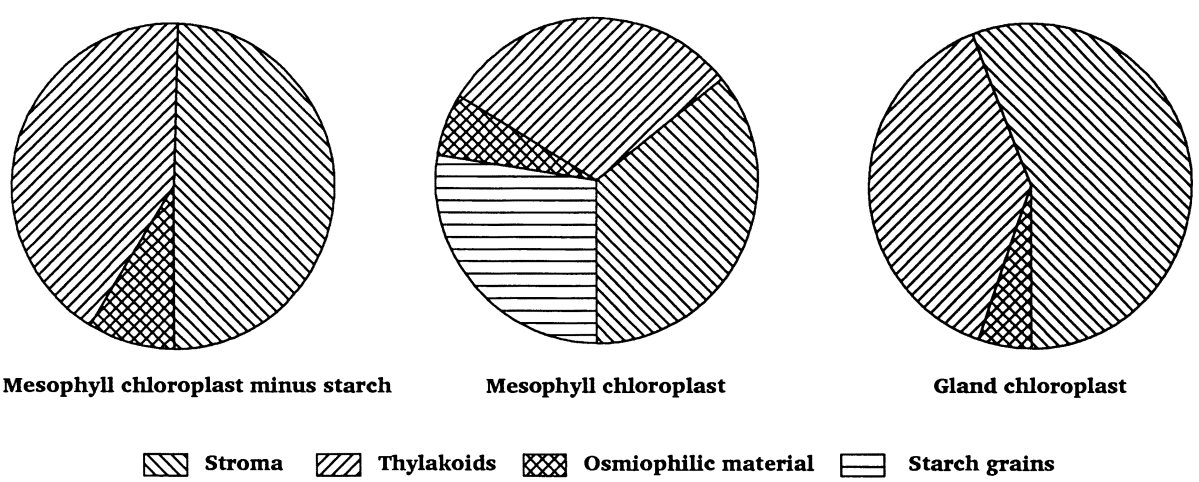

Fig. 9 Comparison of volume densities of chloroplast constituents of plastids from cells of subapical mature cell pairs (see fig. 5) with those of mature mesophyll cells of *Artemisia annua*.

The more amoeboid form of gland cell chloroplasts as compared with the typically ellipsoid chloroplasts of mesophyll tissues may in part result from the absence of a large central vacuole in the gland cells. Compaction between the plasma membrane and tonoplast may contribute to the ellipsoid shape of chloroplasts of mature mesophyll cells.

The stalk cells of resin-secreting glandular hairs of *Beyeria viscosa* have chloroplasts, whereas the cells at the hair tip have amoeboid leucoplasts (Dell and McComb 1978*b*). The amoeboid leucoplasts are ensheathed in an extensive network of smooth ER. These cells are ultrastructurally similar to the apical cells of the *A. annua* capitate gland (figs. 4, 5*A*). The apical or head cells of the glandular hairs of *Newcastelia viscida* (Dell and McComb 1978*b*) and *Eremophila fraseri* (Dell and McComb 1977) also contain amoeboid leucoplasts in an ER network.

The two- and four-cell stages of development of the *A. annua* gland are similar to those of *Cannabis sativa* L.; however, after the next periclinal cell division in *C. sativa,* the apical cells undergo anticlinal proliferation to form a secretory disc (Kim and Mahlberg 1991). The cuticle separates only from the one-cell-thick discoid tier of cells. In *Chrysanthemum* (Vermeer and Peterson 1979*a*), the stalks of the capitate glands are also composed of 10 cells in a biseriate structure, as in *Artemisia* species. In both *Chrysanthemum* and *Artemisia,* the secretory sac is composed of the greatly expanded cuticle of the last three pairs of cells. As in *C. sativa* (Kim and Mahlberg 1991), the cuticle of the secretory sac is significantly thicker than the cuticle of other leaf structures. We did not find this in *A. annua.*

The majority of evidence indicates that the terpenoids generated by capitate glands are produced by the cells of the gland rather than by other cells of the leaf (Mahlberg et al. 1984; Gershenzon et al. 1991; Wagner 1991). While terpenoids can be synthesized outside of plastids (Kleinig 1989), plastids appear to be highly involved in terpenoid production in epidermal glands of plants. Nielsen et al. (1991) found that a nonsecreting line of tobacco lacked chloroplasts in gland cells. Spring and Bienert (1987) found production of terpenoids in sunflower (*Helianthus annuus* L.) capitate glands to be highly dependent on light level, indicating photosynthetic participation in synthesis. Mahlberg et al. (1984) reported that material interpreted as secretory product of *C. sativa* L. gland cells appeared at the plastid surface and migrated through the cytoplasm to accumulate along the cell surface adjoining the secretory cavity between the cuticle bonded to a portion of the primary cell wall and the remainder of the primary cell wall. This is very similar to our interpretation of the apparent movement of osmiophilic material in *A. annua* glands.

An ultrastructural study of terpenoid-producing epithelial cells of *A. campestris* resin ducts showed a slightly different pattern (Ascensão and Pais 1988). During early stages of secretion, somewhat isodiametric plastids with poorly developed thylakoids contained osmiophilic material in the stroma and thylakoids. These plastids were surrounded by smooth ER. Later, the plastid envelope and ER contained osmiophilic material and the plastid had lost internal osmiophilic material. The inner sides of cell walls bordering the resin-duct lumen had ingrowths similar to the reticulations of the inner cell wall of the apical gland cells of *A. annua* (figs. 5*A, B,* 7*D*). Furthermore, the smooth ER-enriched cytoplasm adjacent to the reticulated cell wall was similar to that observed in apical stalk cells (fig. 5*B*).

Dell and McComb (1977) found resin formation and secretion in the cells of glandular hairs of *E. fraseri* to be similar in most respects to what we have described. The only significant differences are that no reticulation of the inner cell walls of secreting cells was noted and that plastids did not appear to be as actively involved in the process.

Differences in plastid morphology between cell pairs of the gland stalk were striking. Of the five cell pairs, only three pairs appeared to have chloroplasts with thylakoids capable of photosynthesis (fig. 5). Several questions are posed that cannot be answered by ultrastructural studies such as presented here. Do all or only certain of the gland cells participate directly in terpenoid production? Three of the five cell pairs of the gland border the secretory cavity, implying that three pairs contribute to filling the cavity with terpenoids. Does each cell type specialize in synthesis of different terpenoids? The morphological differences imply a division of biosynthetic function. Do those cells with photosynthetically active plastids provide substrate for terpenoid production in adjacent cells? These and other questions remain to be answered. Although intact glands can be isolated and studied biochemically (Dell and McComb 1978*a;* Keene and Wagner 1985; Gershenzon et al. 1991; Yerger et al. 1992), no methods are currently available for separating the different cell types of the gland. Determination of the identity of the material in the subcuticular space will require cytochemical methods and/or selective extraction and chemical analysis.

Acknowledgment

We thank Mary V. Duke for her expert technical assistance.

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