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Immunochemical Localization of Tetrahydrocannabinol (THC) in Chemically Fixed Glandular Thrichomes of *Cannabis* (Cannabaceae)

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Monoclonal antibody for delta-9-tetrahydrocannabiol (THC Ab), conjugated with protein A-gold, was employed as a probe to detect THC localization in the gland and subjacent cells of chemically fixed bracts of *Cannabis*. THC was detected in the outer wall of the disc cells, fibrillar matrix, the surface feature of secretory vesicles, and sheath throughout development of the secretory cavity. The probe was absent from vesicles. Label was also present in anticlinal walls of disc cells and walls of dermal and mesophyll cells. Little or no THC Ab was present in disc cells and none were detected in control tissues. This distribution pattern of THC Ab was similar to that in tissues prepared by high pressure cryofixation-cryosubstitution. Consistent association of THC with wall and wall-derived materials suggests that cannnabinoids are synthesized outside the plasma membrane and bound to a wall component, where-upon they are transported to the cavity with wall materials released from the disc cell wall during development of the secretory cavity.

Cannabinoids represent a distinct group of terpenophenolic compounds present only in the genus *Cannabis*. These compounds include the psychoactive component, Δ^9-tetrahydrocannabinol (THC), which accumulates in glands on the plant surface (Lanyon, Turner and Mahlberg, 1981; Turner, Hemphill and Mahlberg, 1978, 1980). These glands consist of a large noncellular secretory cavity subtended by a layer of disc cells which secrete diverse substances into the secretory cavity. Disc cells contain an abundance of plastids associated with the secretory process (Kim and Mahlberg, 1997a). This cavity is formed from the outer wall of disc cells which separates tangentially in its median plane. The outer wall portion, representing the subcuticular wall, and the cuticle form the sheath of the secretory cavity. Both the cuticle and the subcuticular wall increase in thickness during secretory cavity development (Kim and Mahlberg, 1991; Mahlberg and Kim, 1991). The secretory cavity becomes filled with membraned vesicles of lipophilic secretions derived from the disc cells. These secretions and the wall matrix, derived from the disc cell wall, provide materials for the synthesis of cuticular and subcuticular wall components (Mahlberg and Kim, 1992, 1999).

In a previous study of these glands, using an immunoprobe for THC, antibodies for this compound were observed to accumulate in the disc cell wall, sheath, surface feature of secretory vesicles, and fibrillar matrix in the cavity. No antibody was detected in the cytoplasm of the disc cells. This study was conducted on tissues prepared by high pressure cryofixation-cryosubstitution (HPC-CS) to minimize the possible redistribution of cannabinoids during the fixation process (Kim and Mahlberg, 1997b). It was pertinent that the label was associated with the cell wall, the wall-derived fibrillar matrix and the surface feature of vesicles, and did not occur in secretory vesicles in the cavity or in the disc cell cytoplasm. This association suggested that THC may be bound with some wall component, and if so, it may not be extracted by typical chemical fixation (CF). Therefore, in this study we examined the localization of the antibody in tissues prepared by CF to determine whether the THC probe would be retained in the secretory cavity, disc cells, and other cells in a pattern similar to that observed in HPC-CS tissues.

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Materials and Methods

Tissue preparation

Bract segments possessing glandular trichomes at different stages during development were collected from flowering pistillate plants of a Mexican strain of *Cannabis* grown under greenhouse conditions (Ham-

monds and Mahlberg, 1973).

Tissues were fixed for 2 h at 4℃ in 4% glutaraldehyde containing 4% dimethylsulfoxide, rinsed in 50 mM sodium phosphate buffer (pH 7.2), and then postfixed for 1.5 h at room temperature with 2% osmium tetroxide vapor (Kim and Mahlberg, 1991). A short fixation time was used to retain maximal protein activity, although it did not provide optimal fixation for typical organellar components in the cytoplasm. Tissues were washed several times with deionized water and dehydrated in an ethanol-acetone series for embedment at 60℃ in Spurr's resin (1969) and polymerized for 24 h in gelatin capsules at 55℃. Silver-gold sections were cut on an LKB-IV Ultramicrotome and mounted on 200 or 300 mesh uncoated nickel grids.

Preparation of antibody

Monoclonal antibody for THC (THC Ab) was prepared in mouse ascites (11.1H2) by Roche Diagnostics Systems, Inc.

Immunolabeling

Sections mounted on grids were etched with 10% hydrogen peroxide for 45 min, rinsed in deionized water, and floated on 0.56 mM sodium metaperiodate (Craig and Goodchild, 1984). They were rinsed with deionized water, floated for 3 h in PBS containing 1% bovine serum albumin (BSA), followed directly by incubation overnight at 4℃ in anti-THC diluted 1:20 or 1:40 (v/v) in PBS-BSA. Sections were rinsed several times with PBS and floated for 6-8 h on protein A conjugated to 20 nm gold particles (Janssen) diluted 1:20 (v/v) in PBS-BSA. They were rinsed with PBS, PBST containing 1% Triton X-100, deionized water (each step for 3-5 min), and air-dried. Labeled sections were stained with uranyl acetate for 20 min and lead citrate for 5 min, and then subsequently examined with a Philips 300 transmission electron microscope at 60 kV.

Results

Immunolabel in the gland wall and secretory cavity

THC Ab was detected in the outer wall of the disc cells prior to the formation of the secretory cavity (Fig. 1A). Gold particles were more prevalent along the inner zone of the wall adjacent to the plasma membrane than in the subcuticular zone of the wall. No label was detected in the hyaline areas in the outer zone of the wall. Immunolabel remained associated with this wall as the outer zone of wall material appeared to loosen in association with the formation of hyaline areas (Fig. 1B). No label was present in the incipient cavity present between wall zones at the cell juncture of disc cells although label was associated with a fibrillar matrix projecting into this cavity. Label was more or less uniformly distributed along anticlinal walls of adjacent disc cells and their middle lamella. Label was not detected over the plasma membrane or between this membrane and the cell wall, but some grains were present along the inner surface of the cell wall close to the plasma membrane.

Upon enlargement of the secretory cavity, abundant THC Ab was present in the wall facing this cavity (Fig. 1C). It was also present in the subcuticular wall. Cuticle, poorly defined perhaps because of the short fixation procedure, was present above the labeled wall. THC label was associated with aggregates of a fibrillar matrix extending from the wall into the cavity and with sectional views of the matrix in the secretory cavity. In glands with an enlarged secretory cavity, THC Ab was abundant throughout the disc cell wall (Fig. 1D). Gold particles were associated with the wall material adjacent to the secretory cavity and also over dense material in the cavity. Gold particles were distributed more or less uniformly throughout the width of the wall.

In other glands showing a median section for the plasma membrane, gold particles were distributed in the wall region adjacent to, but not over, the plasma membrane (Fig. 1E). No label was evident over hyaline areas in the cell wall or in the secretory cavity. THC Ab was abundant in the disc cell wall in glands with an enlarged secretory cavity (Fig. 1F). Label was also distributed along the anticlinal walls and the middle lamella of contiguous cells. Gold particles were present over the fibrillar matrix which projected into the secretory cavity. No or few gold particles were present in the electron-light region of the secretory cavity.

Immunolabel was distributed throughout the surface feature surrounding vesicles in the cavity (Fig. 2A). In contrast, no gold particles were present in the more or less round secretory vesicles. There was continuity for the distribution of particles in the fibrillar matrix extending from the wall to dense quantities of this material in the secretory cavity (Fig. 2B). Few or no gold particles occurred in electron-light regions of secretory vesicles. Immunolabel was present in the wall of epidermal and parenchymal cells of the bract (Fig. 2C).

Immunolabel in the cytoplasm

Very little THC Ab was present in the cytoplasm of disc cells. Occasionally a gold particle was present adjacent to the plasma membrane (Fig. 1E) or in the subjacent cytoplasm (Fig. 1C and 1D). Similarly, a gold particle was sometimes detectable over a plastid in disc cells prior to secretory cavity development (Fig. 1A and Fig. 1F). Label was occasionally present among ribosomes (Fig. 2A and 2B). No label was detected over the nuclei, vacuoles or Golgi apparati (Fig. 1D).

Controls

No label was detected in *Cannabis* controls treated separately with THC Ab or protein A-gold. No immunolabel

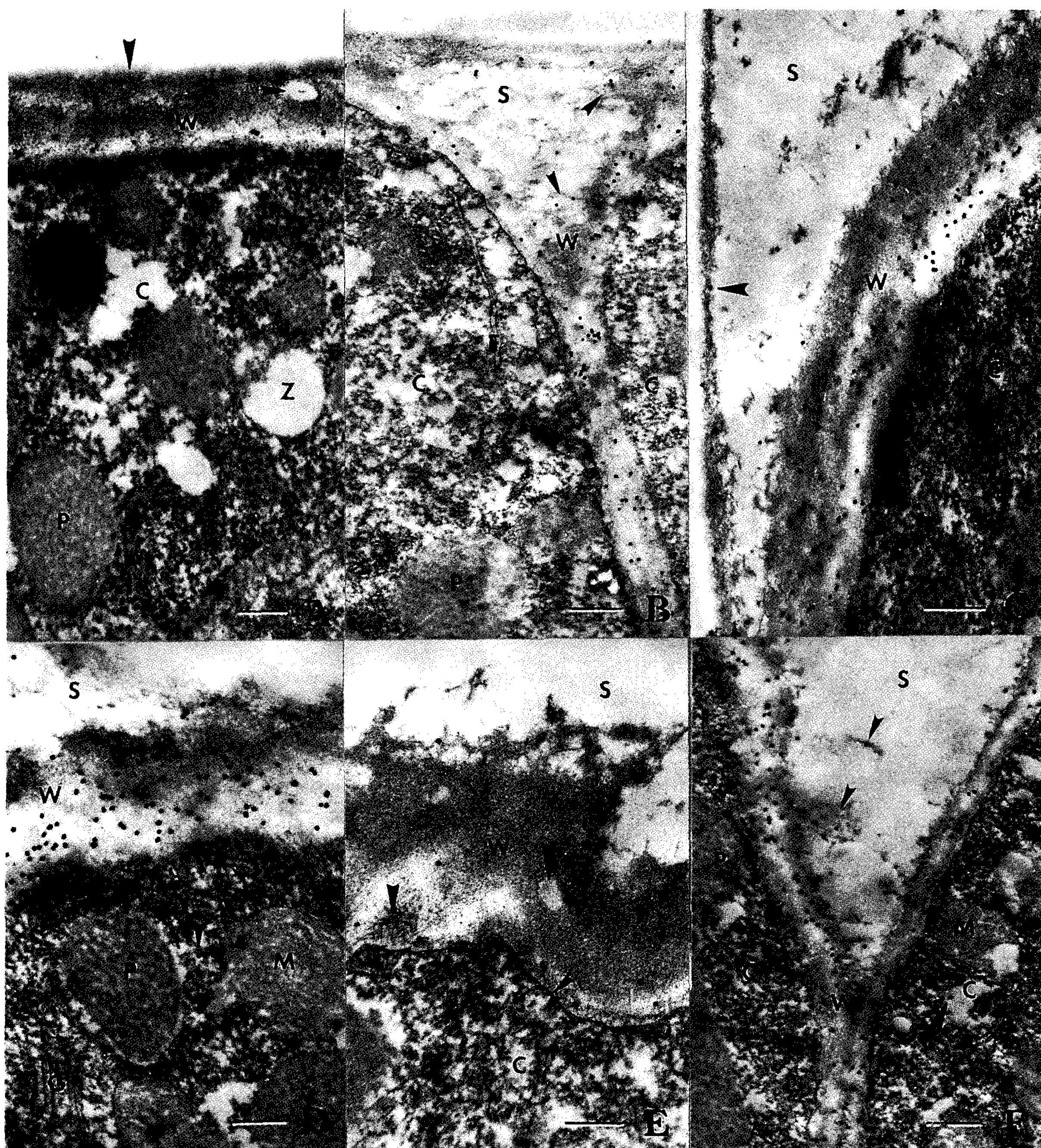

Fig. 1. Immunolabel for THC in glands of *Cannabis*. No extraneous label occurred outside of the gland tissue. A, Disc cell prior to secretory cavity formation showing gold particles in the cell wall. Electron-light hyaline area (arrowhead) is the initial stage in the formation of the secretory cavity. Labeling rarely occurs in the cytoplasm; one gold particle is present over a plastid. Cuticle, large arrowhead. B, Junction region of two disc cells at the time of secretory cavity initiation showing THC label in the outer wall region (above S symbol), and along anticlinal walls between the adjacent disc cell. No label is evident inside the incipient secretory cavity, but is associated with the loose wall matrix (arrowhead) extending into this cavity. Several gold particles are present among ribosomes, but not over endoplasmic reticulum. C, A gland with enlarged secretory cavity showing numerous gold particles in the cell wall. THC label is also evident in the subcuticular wall (as at large arrowhead) and associated with the wall matrix extending into the secretory cavity. One particle is present among ribosomes (small arrowhead). D, Portion of disc cell showing dense THC labeling in the cell wall facing the secretory cavity and in the wall matrix extending into this cavity. One gold particle is present among ribosomes (arrowhead), but no label is detected over the plastid, mitochondrion or Golgi apparatus. E, Disc cell showing THC label distributed in the cell wall. Most particles are in close proximity to the plasma membrane (small arrowhead). A dense region (medium arrowhead) and a hyaline area (large arrowhead) are evident in the cell wall. A gold particle is present over wall matrix extending into the secretory cavity. F, Juncture region between two adjacent disc cells showing THC label distribution in the cell wall and wall matrix (small arrowheads) extending into the enlarged secretory cavity. Many gold particles are located close to the plasma membrane in the cell at the right. A gold particle is evident among ribosomes (large arrowhead) and related to a plastid. C, disc cell; E, ER; G, Golgi apparatus; M, Mitochondrion; P, Plastid; S, Secretory cavity; W, Cell wall; Z, Vacuole. Scale bars=0.2 μm (A, D, E) and 0.3 μm (B, C, F).

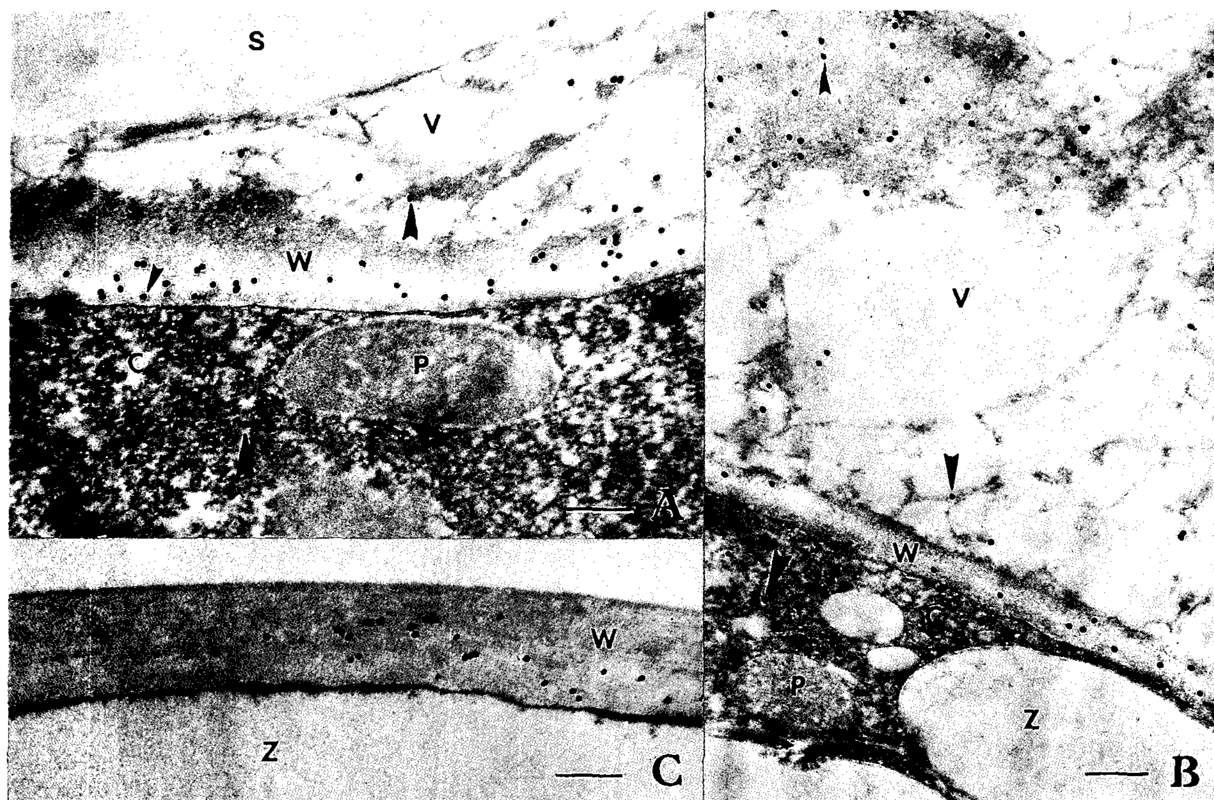

Fig. 2. Immunolabel for THC in glands of *Cannabis*. No label was present outside of the gland tissue. A, Portion of enlarged gland showing immunolabel distribution in the disc cell wall, frequently close (small arrowhead) to the plasma membrane, and associated with the wall matrix (medium arrowhead) extending into the secretory cavity. Very few particles are located over the electron-light areas interpreted to be secretory vesicles. Several gold particles are evident among ribosomes (large arrowhead). B, Disc cell and secretory cavity showing little or no label in the enlarged secretory vesicles, whereas electron-dense contents in the cavity (around small arrowhead) are uniformly labeled. Label identifies THC in the disc cell wall as well as on the wall matrix (medium arrowhead) extending into the secretory cavity. A gold particle is present over a plastid. A few gold particles (as at large arrowhead) occur in the cytoplasm, but not in vacuoles of the disc cell. C, Cell wall of epidermal cell showing the presence of gold label in the wall. V, Secretory vesicle. Scale bars=0.2 µm (A, B) and 0.3 µm (C).

was detected in controls of pea (*Pisum*) leaf tissues treated with the THC Ab protein A-gold probe.

Discussion

A similar pattern of THC Ab distribution was evident in tissues prepared by either CF or HPS-CS. In both procedures the probe was abundant in the outer cell wall of disc cells prior to the formation of the secretory cavity. In glands with a developing and enlarged secretory cavity, THC Ab consistently labeled the disc cell wall, fibrillar matrix, the surface feature of vesicles, and the subcuticular wall and cuticle of the sheath. It was also detected in the cell wall of the epidermis and parenchyma. In contrast, little or no label was present in the cytoplasm of cells. The distribution pattern in tissues prepared by two different techniques suggests that minimal redistribution and, possibly, extraction occurred during tissue processing (Kim and Mahlberg, 1997b). The HPC-CS procedure has been used successfully to preserve proteoglycans and other aqueous-soluble cell components in animal tissues (Hunziker and Schenk, 1984; Hunziker, 1993).

THC in both procedures was not detected in the cytoplasm of disc cells, except for a few spurious grains. Localization of the probe outside the plasma membrane supports an interpretation that cannabinoids are synthesized outside this membrane or in the cell wall and deposited at wall sites (Kim and Mahlberg, 1997b; Mahlberg and Kim, 1999).

Presence of the probe in the wall and wall-derived components in the secretory cavity in tissues prepared by both procedures supports an interpretation that THC and other cannabinoids are bound in some way to a component in the wall. The fibrillar matrix and surface feature of vesicles are derived from the disc cell wall during secretory cavity enlargement. Portions of the cell wall facing the cavity continuously loosen during gland development. They are released into the cavity as the fibrillar matrix. As secretions pass through the disc cell wall and emerge from the wall facing the cavity, each secretion droplet becomes enclosed in a distinctive surface feature upon its release into the secretory cavity. Whereas the probe was detected in the surface feature, it was absent from the vesicle content. Presence of cellulase in the disc cell wall, wall

matrix, surface feature, and subcuticular wall suggests continued modification of wall organization during development of the secretory cavity (Kim and Mahlberg, 1991, 1995, 1997a, b; Mahlberg and Kim, 1991, 1992, 1999). The association of THC with wall and wall derivatives supports an interpretation that cannabinoids are transported into the cavity while bound to wall-derived components released into the cavity, and that they do not enter the cavity as soluble substances. The nature of cannabinoid affinity to wall materials must be examined further.

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