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Leaf anatomy and ultrastructure of the Crassulacean-acid-metabolism plant *Kalanchoe daigremontiana*

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Abstract. Light-microscopic analysis of leaf clearings of the obligate Crassulacean-acid-metabolism (CAM) species *Kalanchoe daigremontiana* Hamet et Perr. has shown the existence of unusual and highly irregular venation patterns. Fifth-order veins exhibit a three-dimensional random orientation with respect to the mesophyll. Minor veins were often observed crossing over or under each other and over and under major veins in the mesophyll. Paraffin sections of mature leaves show tannin cells scattered throughout the mesophyll rather evenly spaced, and a distinct layer of tannin cells below the abaxial epidermis. Scanning electron microscopy showed that bundle-sheath cells are distinct from the surrounding mesophyll in veins of all orders. Transmission electron microscopy demonstrated developing sieve-tube elements in expanded leaves. Cytosolic vesicles produced by dictyosomes undergo a diurnal variation in number and were often observed in association with the chloroplasts. These vesicles are an interesting feature of cell ultrastructure of CAM cells and may serve a regulatory role in the diurnal malic-acid fluctuations in this species.

Key words: Crassulacean acid metabolism (leaf structure) – Cytosolic vesicles – *Kalanchoe* – Leaf ultrastructure and venation.

Introduction

Crassulacean acid metabolism (CAM) is recognized as a photosynthetic pathway distinct from C_3 and C_4. It is known to occur in at least 25 angiosperm families (for recent reviews see Ting 1982; Winter 1985), as well as a few gymnosperm and fern species (Warmbrodt 1984; Winter 1985). Crassulacean acid metabolism occurs primarily in species indigenous to arid regions; it has also been noted in species inhabiting arid microhabitats and in many plants that are popularly termed succulent, characterized by thickened stems or leaves modified for water and acid storage (see Kluge and Ting 1978). In the last two decades, there has been much research into the mechanism of CAM photosynthesis and its relation to solute movement within the leaf and mesophyll (Lüttge et al. 1982; see Uribe and Lüttge 1984, for review) but only recently has a comprehensive overview of the physiological and biochemical mechanisms of CAM emerged (see books and reviews by Kluge and Ting 1978; Osmond 1978; Ting 1985; Winter 1985).

Information on the leaf anatomy of CAM species and its relation to the CAM pathway is, however, quite limited (see Gibson 1982). Mesophyll cells of CAM plants are not usually differentiated into palisade and spongy parenchyma (Kluge and Ting 1978). There tends to be less free air space between mesophyll cells of CAM species than between the mesophyll cells of C_3 and C_4 plants (Lüttge and Ball 1977). Bundle-sheath cells often differ little in appearance from the surrounding mesophyll (see Kluge and Ting 1978 for a general overview of CAM anatomy). Mesophyll cells in *Mesembryanthemum crystallinum* were found to be highly parenchymatous, with a peripheral band of cytoplasm between the plasmalemma and tonoplast (von Willert and Kramer 1972). Warmbrodt (1984), studying the fern *Pyrrossia longifolia*, found that "numerous small vacuoles, all of which are devoid of visible contents, occur in the parietal layer of cytoplasm." He also found a high number

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Abbreviations: CAM = Crassulacean acid metabolism; SEM = scanning-electron microscopy; TEM = transmission-electron microscopy

of plasmodesmata connecting the mesophyll and bundle-sheath cells.

Ultrastructural studies of mesophyll in CAM cacti have shown that chloroplasts tend to accumulate large deposits of starch and-or plastoglobuli (Thomson and Platt 1973; Kapil et al. 1975; Teeri and Overton 1981). Storage carbohydrate fluctuates diurnally, consistent with CO_2 fixation in CAM (Kluge and Ting 1978; De Santo et al. 1984). Mitochondria tend to be the most abundant organelles in mesophyll cells, followed by chloroplasts (Warmbrodt 1984). Organelle ontogeny and abundance in CAM mesophyll and apical meristems has been examined in the genus *Kalanchoe* (Gifford and Stewart 1967; Kapil et al. 1975) where single-membrane spherosomes were found in the apical cells. The authors also noted the presence in the proplastids of phenolics which were transported into vacuoles as the plastids matured. Proplastids of *Kalanchoe* remained immature much longer than in the C_3 species studied.

Kalanchoe daigremontiana is one of the most extensively investigated CAM species in terms of the biochemistry and physiology of the CAM pathway (Lüttge et al. 1982); however, little anatomical work has been attempted on this species (Gifford and Stewart 1967; Kramer and von Willert 1972). In this paper we describe anatomical characteristics of this species in the attempt to relate structural features to the biochemistry of CAM.

Material and methods

Kalanchoe daigremontiana. Hamet et Perr. plants were grown from plantlets under a 12-h photoperiod (350 μM photons $\cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation; Westinghouse Corp., New York, N.Y., USA 185-W cool-white fluorescent, and 25-W incandescent lights) in a growth chamber maintained at 25° C day/20° C night. Leaf clearings were prepared from six- to nine-month-old plants. One leaf from the first through fourth visible leaf pairs from the apex of four different plants was excised, washed in deionized water, cut into 3–5-cm^2 pieces, and immersed in 70% acetone for two weeks (with fresh solution changes every 48 h) to remove chlorophylls. The leaf sections were then washed with 50% and 30% acetone (15 min each), followed by deionized water, and immersed in 5% NaOH for 5 d with daily changes of the solution. Sections were stained with 0.1 M potassium permanganate for 5 min and repeatedly washed in deionized water to remove excess stain; after this, they were infiltrated in a series of water-glycerol mixtures of 50, 75, 100% glycerol for 15 min each. Tracings and micrographs were obtained by observing and photographing wet mounts in 100% glycerol.

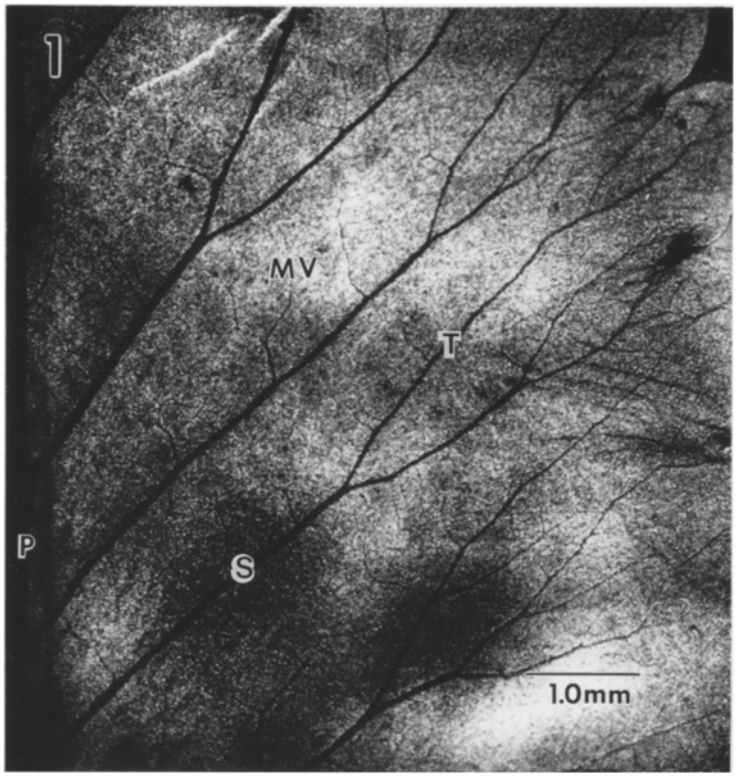
Material for light microscopy was harvested from similar plants. Leaves of various stages of development were collected and fixed in a mixture of 4% (v/v) formaldehyde, 5% (v/v) acetic acid and 50% (v/v) ethanol for 48 h at room temperature. Samples were then dehydrated in an ethanol series, infiltrated in xylenes, then paraplast (Lancer Inc., St. Louis, Mo., USA). Sections (10 μm thick) were obtained on a rotary microtome (American Optical Co., Buffalo, N.Y., USA) using steel knives and stained with either safranin-fast green, crystal violet-congo red, or periodic acid Schiff's reagent-mercurobromphenol blue. Light micrographs were taken on a Leitz (Rockleigh, N.J., USA) Ortholux microscope using a Orthomat camera system and Kodak Technical Pan black & white film ASA 100 (Eastman-Kodak, Rochester, N.Y., USA).

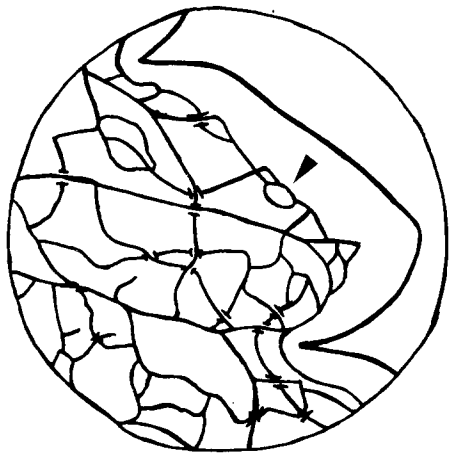
Material for scanning electron microscopy (SEM) was obtained from similar plants, harvesting both of the fourth visible leaves from the apex. Cross and longitudinal sections 5–10 mm^2 of veins and mesophyll were cut from different areas of the lamina, fixed in 0.5-strength Karnovsky (1965) solution overnight at 4° C, and post-fixed with 1% OsO_4 in 25 mM cacodylate, pH 7.1, for 2.5 h also at 4° C. The samples were dehydrated in an ethanol series, freeze-fractured in liquid nitrogen, critical-point dryed, mounted on stubs and gold-sputter coated. All micrographs were obtained using an ETEC Autoscan U-2 scanning electron microscope (Hayward, Cal., USA) at 20 kV.

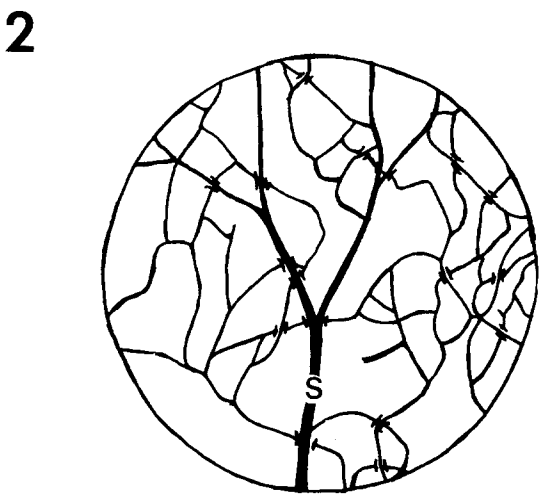
Material for transmission electron microscopy (TEM) was taken from similar areas as described for the SEM. Pieces (1–2 mm^2) were fixed overnight in 2% paraformaldehyde plus 2.5% glutaraldehyde in 50 mM 1,4-piperazine di ethanesulfonic acid (Pipes) pH 7.0, at 4° C. They were then post-fixed with 1% OsO_4 in 25 mM cacodylate, pH 7.1, for 2.5 h at room temperature. Samples were dehydrated in an acetone series, and infiltrated with Spurr's (1969) resin. Silver-gold sections were obtained using glass knives (Reichert OmU2 ultramicrotome). Sections were post-stained with 2% uranyl acetate for 15 min followed by Reynold's (1963) lead citrate for 5 min. All micrographs were made using a Hitachi (Tokyo, Japan) HS-8 transmission electron microscope at 50 kV.

Results and discussion

The description of leaf venation characteristics in *Kalanchoe blossfeldiana* follows the system of classification of Hickey (1973). A single, large midvein (primary vein) traversed the length of the lamina. Secondary, tertiary, and quaternary venation was cladodromous; veins branching off the primary one freely ramified and dichotomized towards the margin. The angle of attachment of secondary veins tended to become smaller with distance from the petiole (Fig. 1), and these veins were often curved concavely towards the margins. Branching was mainly oriented towards the margin of the lamina, with occasional minor veins branching off perpendicular to the major veins (Fig. 1). Fifth-order venation was random reticulate and aplanar, exhibiting a three-dimensional lattice. The veins freely traversed adaxial to abaxial areas of the mesophyll, often passing over or under larger veins and over and under each other (Fig. 2). The fifth-order veins tended to anastomose and form continuous loops (areoles) (Fig. 3) along the margin. The areoles were therefore irregular and of random size, increasing in area with leaf expansion. The aplanar arrangement of the minor veins may be a consequence of the thickness of the lamina, and







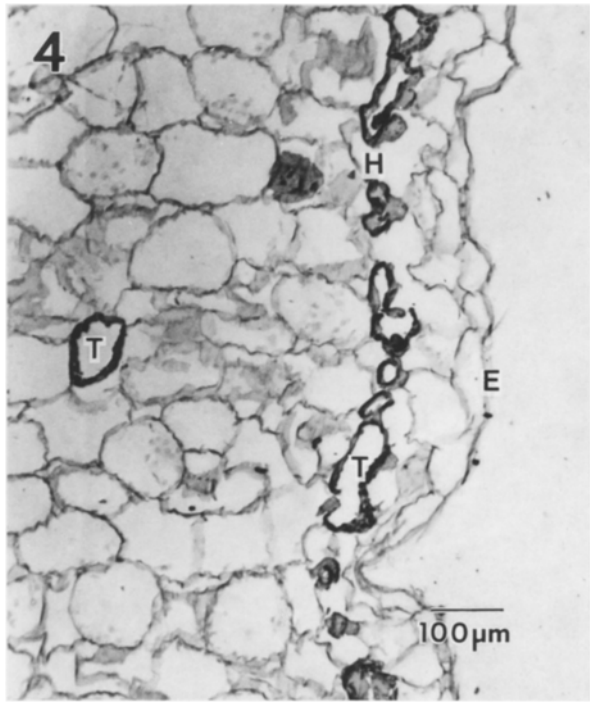


Fig. 1. Clearing light micrograph of the first visible leaf from the apex in *Kalanchoe daigremontiana*. Venation patterns are already determind, minor veins branch freely off secondary (*S*) and tertiary (*T*) veins. *P* primary vein; *MV* minor vein. × 15

Fig. 2. Projection-microscope leaf tracing showing three-dimensional venation pattern. *Broken lines* indicate the vein travels underneath a vein (*continuous lines*). The secondary vein (*S*) dicotomizes towards the margin; minor veins exhibit a three-dimensional lattice traveling over and under each other, and the larger veins present. × 20

Fig. 3. Projection-microscope leaf tracing of the edge of the lamina where fifth-order veins form areoles (*arrow*). × 20

Fig. 4. Light micrograph of mature leaf cross section. Abaxial epidermis (*E*), hypodermis (*H*), and mesophyll tissue. *T* tannin cells. × 100

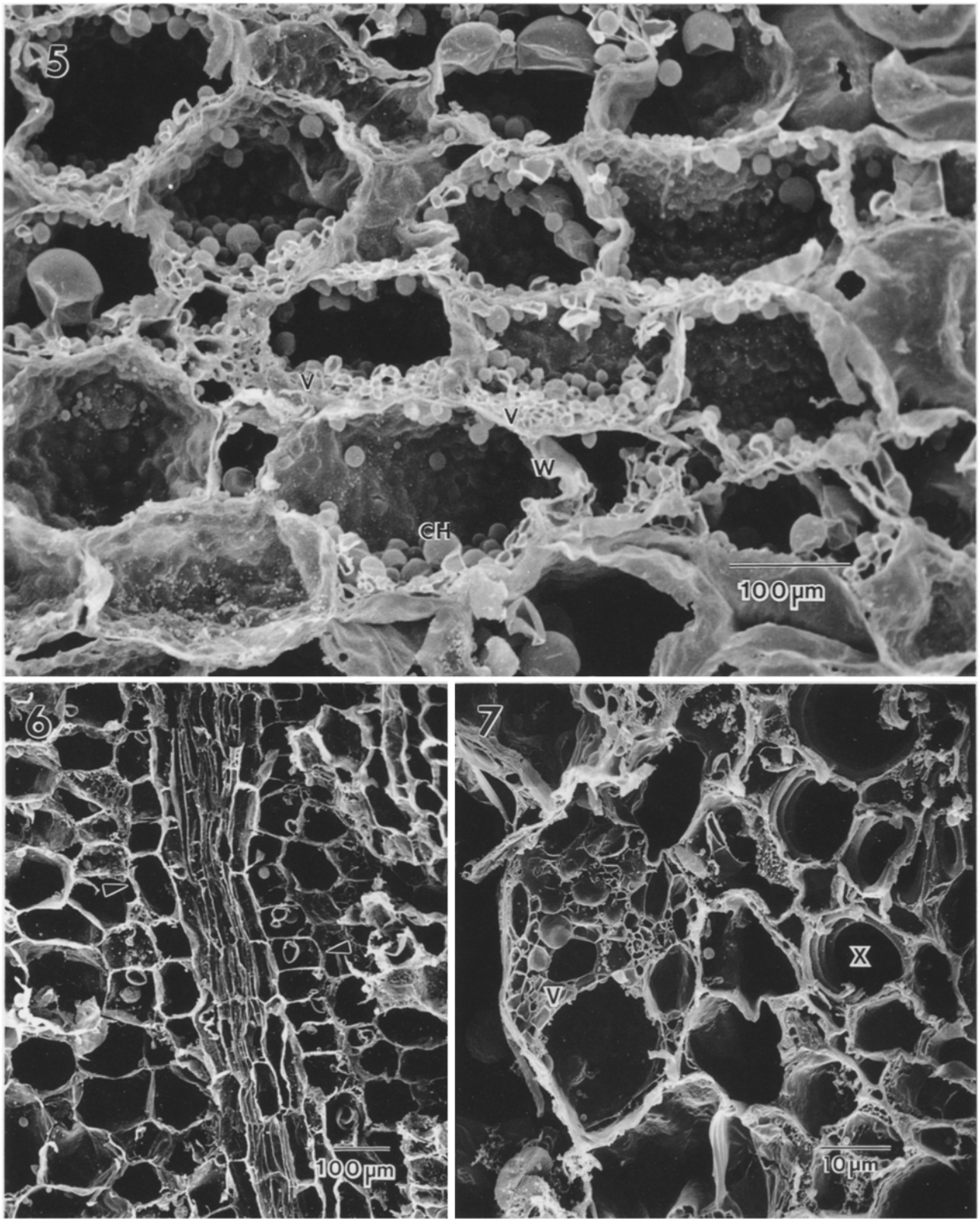


Fig. 5. Scanning electron micrograph of a cross section of the mesophyll of a mature leaf of *Kalanchoe daigremontiana*. *CH* chloroplast, *V* vesicles, *W* cell wall. ×210

Fig. 6. Scanning electron micrograph of a longitudinal section of a mature leaf with a tertiary vein and bundle-sheath cells (*arrows*). Bundle-sheath cells are clearly differentiated from surrounding mesophyll. ×100

Fig. 7. Scanning electron micrograph of a mature leaf cross section showing annular secondary wall thickenings of the xylem (*X*). Numerous vesicles (V) are present in the mesophyll cell on the left. ×1200

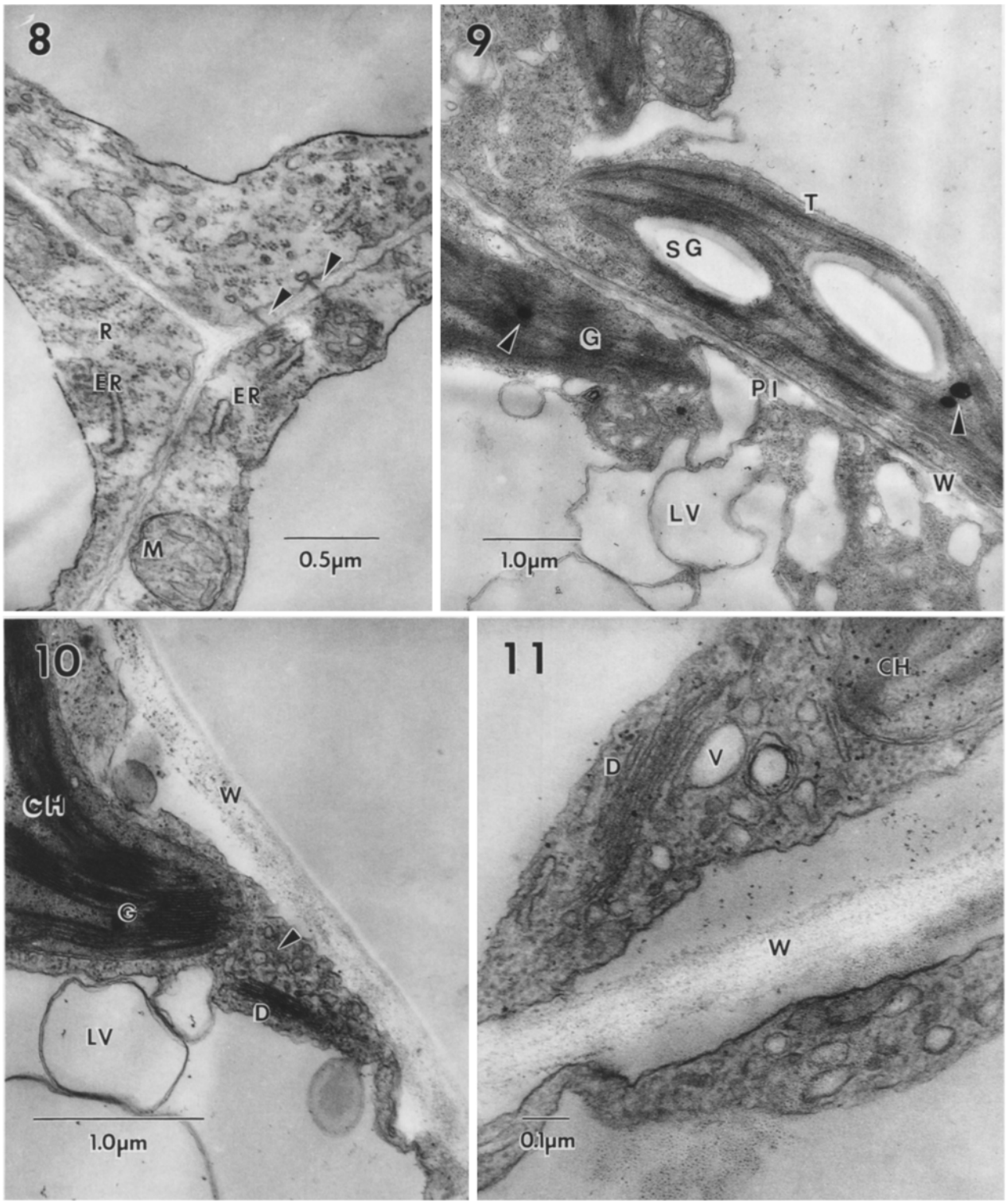


Fig. 8. Transmission electron micrograph of vascular-bundle cells showing of *Kalanchoe daigremontiana* endoplasmic reticulum (*ER*), mitochondria (*M*), plasmodesmata (*arrows*), and ribosome clusters (*R*). × 31 700

Fig. 9. Transmission electron micrograph of two adjacent mesophyll cells. Chloroplasts contain grana stacks (*G*), two large starch grains (*SG*), and plastoglobuli (*arrows*). *W*, cell wall; *PL*, plasmalemma; *T*, tonoplast; *LV*, large vesicles appressed to the tonoplast and protruding into the vacuole. × 16 500

Fig. 10. Transmission electron micrograph of dictyosomes (*D*) producing small cytosolic vesicles. *CH*, chloroplast; *G*, grana stack; *arrow:* small vesicles; *W*, cell wall; *LV*, large vesicles. × 28 500

Fig. 11. Transmission electron micrograph of enlarging vesicles produced by the dictyosome (*D*). *CH*, chloroplasts; *W*, cell wall; *V*, vesicles. × 73 500

may offer greater structural support to the mesophyll.

Epidermal cells were rectangular and possessed a thickened cuticle. The upper epidermis was one to two cells thick, the lower two to three cell layers (Fig. 4). The layer immediately below the abaxial epidermis was mesophyll tissue, below which was a hypodermis one to two cells thick. The hypodermal layer consisted of evenly spaced cells which contained tannin deposits (determined by a black, dense precipitate caused by potassium permanganate or OsO_4 oxidation, or their preferential staining by cationic dyes) in the central vacuole, and also had tannins deposited in the walls. Additional tannin-containing cells were somewhat sparsely and evenly distributed throughout the mesophyll (Fig. 4).

Veins of all orders had the normal xylem-phloem adaxial-abaxial arrangement, with the midveins containing a central cambial region. Developing sieve elements with protein bodies (P-bodies) were often observed in large expanded leaves (not shown). Bundle-sheath cells were elongated and rectangular compared to mesophyll cells (see Fig. 5 for mesophyll, and longitudinal view of vein in Fig. 6). All veins observed were bounded by a bundle sheath. Tracheids, sieve tubes, companion cells and phloem parenchyma were present in all vein classes, even close to the vein endings. Tracheids and vessels had annular secondary wall thickenings (Fig. 7). Vascular parenchyma cells contained a large number of mitochondria and often contained plastids with developed grana and plastoglobuli (not shown). Starch grains were not observed in the plastids of these cells. These features are similar to those described for the vascular parenchyma of *Pyrrossia longifolia* (Warmbrodt 1984). A large number of ribosomes and rough endoplasmic reticulum was present in the vascular parenchyma cells and indicated a high level of metabolic activity in these cells, including those in expanded leaves. Plasmodesmata were infrequently observed connecting cells bordering the vascular bundles (Fig. 8).

Mesophyll cells were thin-walled, 80–100 μm in length, oblong to spherical in shape, and highly vacuolate. The cells were densely packed, with little intercellular air space, and were not differentiated into palisade or spongy parenchyma (Figs. 4–6). In contrast to *Pyrrossia longifolia*, plasmodesmata were rarely encountered between mesophyll cells. This may indicate that a substantial portion of the intercellular transport of photoassimilates in the mesophyll of *Kalanchoe* is through the apoplast, and may also partially explain the fifth-order venation observed. Further study in this area is needed before any conclusions can be drawn. Nuclei were irregular in outline, containing a single, large nucleolus. The chloroplasts, 6–12 μm in length, usually contained one to five large starch grains (depending on the plane of the section), varying amounts of plastoglobuli, and distinct grana (Figs. 9, 10). Mitochondria were the most numerous organelles, the number and size varying greatly. The highest numbers were found in developing companion cells and vascular parenchyma (Fig. 8). Cytosolic vesicles similar to those described by Warmbrodt (1984) were observed throughout the cytoplasm in both SEM and TEM preparations (Figs. 5, 9–11) and tended to cluster around the chloroplasts. Dictyosomes were occasionally found near chloroplasts producing tiny vesicles (Fig. 10) which enlarge, possibly by coalescing, into the larger cytosolic vesicles (Figs. 9–11). Most dictyosomes observed formed vesicles on the plasmalemma side, though this was not always the case. Larger vesicles in association with the chloroplasts, and appressed to the tonoplast were often observed in sections (Figs. 9–11). Present evidence indicates that these cytosolic vesicles are produced by the dictyosomes and enlarge into vesicles of sufficient size to cause substantial changes in the outline of the tonoplast of the central vacuole (see Figs. 10, 11). These cytosolic vesicles exhibited the same ATPase activity which is localized on the cytoplasmic surface of the tonoplast of the central vacuole (Balsamo and Uribe 1986, 1987; see Figs. 1, 7, 10 in latter paper). It is possible that these vesicles perform some regulatory or transport function. The increase in the surface-to-volume ratio provided by the presence of many vesicles would make it possible to sequester rapidly malic acid synthesized nocturnally, thus reducing the extent of cytoplasmic acidification in CAM, or these larger vesicles may facilitate movement of malic acid from the cytosol to the central vacuole. Further studies are required to test these hypotheses.

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