Peperomia leaf cell wall interface between the multiple hypodermis and crystal-containing photosynthetic layer displays unusual pit fields

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Received: 17 January 2012 Accepted: 28 February 2012 Published electronically: 25 April 2012

• *Background and Aims* Leaves of succulent *Peperomia obtusifolia* (Piperaceae), and its related species, contain a large multilayered hypodermis (epidermis) subtended by a very small single-layered photosynthetic palisade parenchyma, the latter containing spherical aggregates of crystals called druses. Each druse is in a central vacuole surrounded by chloroplasts. All hypodermal cell walls are thin, except for thick lowermost periclinal walls associated with the upper periclinal walls of the subtending palisade cells. These thick walls display 'quilted' impressions (mounds) formed by many subtending palisade cells. Conspicuous depressions occur in most mounds, and each depression contains what appear to be many plasmodesmata. These depressions are opposite similar regions in adjacent thin palisade periclinal walls, and they can be considered special pit fields that represent thin translucent regions ('windows' or 'skylights'). Druses in the vacuoles of palisade cells occur below these pit field regions and are surrounded by conspicuous cytoplasmic chloroplasts with massive grana oriented perpendicular to the crystals, probably providing for an efficient photosynthetic system under low-intensity light.

• *Methods* Leaf clearings and fractures, light microscopy and crossed polarizers, general and histochemical staining, and transmission and scanning electron microscopy were used to examine these structures.

• *Key Results* Druses in the vacuoles of palisade cells occur below the thin pit field regions in the wall interface, suggesting an interesting physical relationship that could provide a pathway for light waves, filtered through the multiple hypodermis. The light waves pass into the palisade cells and are collected and dispersed by the druses to surrounding chloroplasts with large grana.

• *Conclusions* These results imply an intriguing possible efficient photosynthetic adaptation for species growing in low-light environments, and provide an opportunity for future research on how evolution through environmental adaptation aids plants containing crystals associated with photosynthetic tissues to exist under low-light intensity and with other stresses.

Key words: Chloroplasts, crystals, druses, multiple hypodermis, leaves, photosynthetic layer, *Peperomia*, interface pit fields.

INTRODUCTION

Many photosynthetic organisms represented by algae, ferns, gymnosperms and angiosperms (Arnott and Pautard, 1970; Horner et al., 2012) normally produce solitary, multiple and aggregated calcium oxalate crystals in the vacuoles of certain cells that occur in different organs, if present, depending on the species. In some cases, this may be extreme, depending on the environment (Braissant et al., 2004). Plant crystals have been reported many times in the literature, dating back to the late 1600s (Leeuwenhoek, 1675; McNair, 1932; Metcalfe and Chalk, 1950; Metcalfe, 1983; Prychid and Rudall, 1999). Because of their striking appearance anatomically, especially when viewed between crossed polarizers using light microscopy (LM), and the fact their function is still not clearly defined, researchers continue to seek plant systems and methodologies to help explain what role(s) they play in a plant's life (Franceschi and Horner, 1980; Horner and Wagner, 1995; Franceschi and Nakata, 2005).

Apart from some obvious examples where crystals seem to serve as protection against predators, such as the stinging nettle plants (*Tragia*; Thurston, 1976) and members of the Araceae such as *Dieffienbachia* (Gardner, 1994), there seems to be little agreement about other possible functions for the crystals (Zindler-Frank, 1976; Ilarslan *et al.*, 2001; Nakata, 2003; Franceschi and Nakata, 2005). This quandary provides an opportunity to explore plant crystal function in various suitable systems.

Since all green leaves are exposed to sunlight and contain photosynthetic cells with chloroplasts to capture the sun's radiation, it is reasonable to expect that certain leaf architectures may have evolved in ways to function optimally under lowlight and water availability conditions. This is true for plant species that inhabit understoreys where canopy foliage can reduce light reaching the plants and where seasonal or periodic fluctuations in availability of water occur. Crystals commonly are found in leaves of a wide variety of species and display a variety of forms and macropatterns that are species and taxon specific (Lersten and Horner, 2000, 2007, 2008, 2009, 2011; Cervantes-Martinez *et al.*, 2005; Horner *et al.*, 2009, 2012). Their presence in some leaves raise the question as to whether they have any functional significance beyond being of taxonomic importance.

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ANNALS OF

BOTANY

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Two closely related genera, Peperomia and Piper (Piperaceae), share similar pantropical geographic ranges and often grow under low-light conditions with variable water availability. The leaf anatomy of these two genera, however, is significantly different from each other but both contain various types of crystals associated with their leaf chlorenchyma tissues (Christensen-Dean and Moore, 1993; Horner et al., 2009, 2012). With respect to the genus Peperomia, several studies have suggested a light gathering and reflection role for the crystals in its leaves (Schüroff, 1908; Franceschi and Horner, 1980; Kuo-Haung et al., 2007; Horner et al., 2009), and for some Piper species (Horner et al., 2012). Apart from the studies of Kuo-Huang et al. (2007) on Peperomia glabella and of Gibeaut and Thomson (1989a) on Peperomia obtusifolia leaves, there has been no additional information regarding the anatomical and ultrastructural features of crystal-containing palisade parenchyma cells and associated chloroplasts in other taxa (Lichtenthaler et al., 1981). This latter feature, coupled with these species having been identified with different photosynthetic mechanisms [C₃, crassulacean acid metabolism (CAM) and/or CAM-cycling) (Virzo de Santo et al., 1983; Ting, 1985; Sipes and Ting, 1985; Nishio and Ting, 1987; Ting et al., 1994), suggests that further research is warranted. Therefore, the purpose of this study is to provide a closer look at the interface relationship between the multilayered hypodermis and palisade parenchyma in P. obtusifolia as the basis for additional studies dealing with crystal-plastid involvement in plants growing under low-intensity conditions.

MATERIALS AND METHODS

Plants of *Peperomia obtusifolia* A.Dietr. (Piperaceae), growing in the Bessey Hall rooftop greenhouse on the Iowa State University campus, were used in this study (Fig. 1A). Cuttings were made to increase the number of plants for use in later studies. The uniformly green leaves were processed in several ways to observe and understand the leaf anatomy and ultrastructure, and the type/shape and location of the crystals.

Leaf clearings

Circular leaf punches using a small-diameter cork borer were made and placed in a multiple compartment tray (Horner and Arnott, 1961), and processed according to Lersten and Horner (2011) to remove all cellular contents except the cell walls and inorganic crystals. The cleared punches were mounted on slides in Permount (www.fishersci.com), coverslipped and viewed between crossed polarizers using an Olympus BX40 compound light microscope (www.olympus.com) fitted with a Zeiss Axioplan colour MRc digital camera (www.zeiss.com).

Vibratome and paraffin sections

Fresh leaf segments were cut into 45 μ m thick sections with a vibratome (tpi-3000; www.tedpella.com). They were mounted in deionized water on slides, coverslipped and immediately viewed and photographed with the same microscopic system.

Leaf punches were also fixed in formalin-acetic acidethanol (FAA; Ruzin, 1999) for 24 h at room temperature. These punches were dehydrated through an ethanol series (25, 50, 70, 95, 100 and 100 %), transferred to xylene and infiltrated and embedded with Paraplast paraffin (54–56 °C mp; www.fishersci.com). The 10 μ m thick cross- and paradermal sections were cut with a steel knife, and mounted on glass slides. Sections were deparaffinized and brought to water where they were stained either with aqueous 1 % chlorozol black E (for general contrast enhancement) or with the aqueous periodic acid–Schiff (PAS) technique (Ruzin, 1999) (for contrast enhancement of water-insoluble polysaccharides). Sections were dehydrated through an ethanol series to xylene; Permount was added along with coverslips. Sections were viewed and photographed with the same light microscopic system.

Scanning electron microscopy (SEM)

Leaf punches fixed in FAA for 24 h were dehydrated from 50 % ethanol through 100 % ethanol: some punches were placed in Parafilm (www.fishersci.com) pillows filled with 100 % ethanol, sealed, frozen in liquid nitrogen and fractured in cross-section. Fractured pieces were placed back into 100 % ethanol. They were treated twice more with ultrapure 100 % ethanol and critical point dried (Denton; www.azom.com) using liquid carbon dioxide. Whole punches were sandwiched between two SEM aluminum stubs that had double-sided sticky taped end surfaces; the stubs were pressed together and split apart so that each stub contained a somewhat paradermal and complementary portion of the torn leaf punch. The fractured punches were mounted vertically on aluminium stubs, fracture side up. Silver paint was applied around the torn leaf punches and at the bases of the fractured leaf punches. All mounted specimens were sputter coated (Denton; www.azom.com) with about 10 nm of palladium:gold (80:20). Specimens were viewed and imaged with a JEOL 5800 scanning electron microscope (www.jeol.com) at 15 kV.

Transmission electron microscopy (TEM)

Small, 1-mm diameter leaf punches were fixed with 2 % paraformaldehyde/2 % glutaraldehyde in a 0·1 M cacodylate buffer (pH 7·24) at 4 °C for 24 h. The punches were buffer washed three times for a total of 1 h, post-fixed in 1 % osmium tetroxide in the same buffer for 1 h, dehydrated through an ethanol series to ultrapure ethanol and embedded in Spurr's resin (hard). Diamond knife-cut sections 60–90 nm thick were placed on copper grids and post-stained with lead and uranium. A JEOL 1200 transmission electron microscope (www.jeol.com) was used to view and capture the images on DuPont Cronar high-resolution film. Processed negatives were tiff digitized. All digitized images were processed in Adobe PhotoShop CS5 and collaged in Adobe Illustrator CS5 (www.adobe.com).

RESULTS

Mature *P. obtusifolia* leaves are succulent and measure >1 mm in thickness (Fig. 1B). They consist of two single-layered epidermises (upper, adaxial; and lower, abaxial), a multilayered hypodermis (i.e. adaxial multiple epidermis by other authors) below the upper epidermis, a single small subtending palisade parenchyma (i.e. median mesophyll by other authors)



FIG. 1. *Peperomia obtusifolia* whole plant, and light (LM) and scanning (SEM) electron micrographs of portions of leaves. (A) Greenhouse plant. (B) SEM cross-section fracture showing different tissues (UE, upper, adaxial epidermis; H, multiple-layered hypodermis; PP, single-layered palisade parenchyma; SP, multiple layered spongy parenchyma; LE, lower, abaxial epidermis; VB, vascular bundle). (C) LM paradermal view of clearing between crossed polarizers focused at the palisade parenchyma layer containing druses. Some druses are out of focus due to non-planar palisade parenchyma. (D) LM paraffin cross-section viewed between partially crossed polarizers accentuating druses in a non-planar palisade parenchyma. Note that all but one druse are located near the common wall with the hypodermis. (E) SEM cross-section fracture of palisade parenchyma wall common with the palisade parenchyma. The arrow identifies one visible druse in a palisade parenchyma. The majority of mounds have one or more pits. (G) SEM of one pit showing blebs purported to be severed plasmodesmata. (H) SEM cross-section fracture through a single pit. The thick hypodermal wall is identified with a vertical green bar. Scale bars: (A) = 5 cm; (B) = 250 μm; (C) = 50 μm; (F) = 10 μm; and (G, H) = 2 μm.

immediately below this hypodermis and a multilayered spongy parenchyma (i.e. lower mesophyll by other authors) in which the venation resides (Fig. 1B). Cleared and paraffin- and vibratomesectioned leaves show that calcium oxalate crystal aggregates, called druses, occur in the typically single-layered palisade parenchyma (Fig. 1C), normally one druse per cell throughout the lamina. This single layer undulates slightly so that it is not planar but follows the bases of the lowermost hypodermal cells (Fig. 1D, E). There are no other crystals in the leaves of this species so it has a crystal macropattern designated as DU-/- (D. druse: U. uniform layer of druses: -/-, neither prisms nor raphides are present in spongy parenchyma) by Horner et al. (2009, 2012). This crystal macropattern is predominant for the genus Peperomia. The other two major druse macropatterns represented in the genus are: species with larger druses over the veins (DUVbig) and smaller druses in the lamina or areole (Asmall) regions (DUVbigAsmall-/-); and druses only over the veins (DR-/-) (Horner et al., 2009, 2012). There are variations of these three macropatterns.

The purpose of this study was to focus on the interface region between the lowermost hypodermal cells and the singlelayered palisade parenchyma, containing the druses, that subtends it and is physically attached to it. Figure 1B provides an overview of this region that is relatively small, compared with the remainder of the leaf anatomy.

The FAA fixation provides relatively well-preserved structures except for the hypodermal protoplasts that have extremely large vacuoles (probably > 80% of the cell volume). The basal hypodermal cells in paradermal section are somewhat flattened or slightly curved, and vary in size, measuring about 100 µm across at their bases. The hypodermal protoplasts are severely plasmolysed due to their tonicity and the FAA fixative used, but, as such, expose the basal walls for direct observation of their inner wall surfaces (Fig. 1F). Each large basal hypodermal cell wall is in direct contact with many smaller palisade parenchyma cells (see later) that are delineated as 'mounds' in these basal walls (Fig. 1F). The majority of hypodermal wall mounds have one or more obvious depressions or pits of different sizes (Fig. 1G). The depths of the pits measure about 0.6 µm (Fig. 1H), whereas the adjacent palisade parenchyma wall is only about 0.1 µm thick. In each pit there are what appear to be membrane-like protrusions that seem to be severed plasmodesmata connecting the basal hypodermal cell with the adjacent subtending palisade cell (Fig. 1G).

In paradermal paraffin sections these mounds are clearly stained and the pit field regions in each mound are either not stained or only lightly stained. This is verified when using either chlorozol black E (Fig. 2A) or PAS (Fig. 2B) staining procedures, suggesting that only the middle lamella is present and serves as the partition between them. The size and number of pit fields vary per mound, and the majority of mounds display pits (Fig 2C). These same results are observed in three other greenhouse *Peperomia* species observed in similar sections (not shown; *P. clusifolia, P. prostrata* and *P. subpeltata*).

When fractures of the wall interface are observed from the palisade parenchyma side in regions where the palisade parenchyma protoplasts are missing, either remnants of the protoplast plasmalemma remain (Fig. 2D, E) or they are missing altogether, exposing this upper wall and thin pit fields with protrusions. The latter are interpreted as plasmodesmata remnants (Fig. 2F, G) similar to those observed in the basal hypodermal wall pit fields opposite them.

Oblique (almost paradermal) SEM fractures through the hypodermal-palisade parenchyma wall interface show the hypodermal wall mounds and the subtending palisade parenchyma cells and their druses (Fig. 2H). The druses are about the same diameter as measured in both LM and SEM images (average diameter = $8.4 \mu m$). They are basically spherical in shape and consist of many individual crystals each with several visible facets (Fig. 2I). These crystals are bound together by an organic nucleation centre (not shown; Horner and Wagner, 1980, 1995). The druses are composed of the elements calcium and oxygen, as determined by X-ray energy dispersive analysis with SEM, and they are not subject to dissolution with 5 % acetic acid (H.T. Horner, unpubl. res.). Based on druses analysed in this way in other Peperomia and non-Peperomia species, the crystal aggregates are deemed to be calcium oxalate.

The palisade parenchyma, consisting of a single layer, is made up of 'U'-shaped cells that are on average about 13 μ m in diameter and 32 μ m in length. Given the average diameters of the basal hypodermal cells (100 μ m) and palisade parenchyma cells (13 μ m), and taking into account palisade parenchyma cell wall thicknesses and intercellular spaces, a single hypodermal cell could be in contact with as many as 50–60 palisade parenchyma cells.

The top of each palisade parenchyma cell is slightly arched, whereas the base of each cell is rounded, as shown by the fractured exposed protoplasts (Fig. 2J). Longitudinal fractures and sections show each protoplast consisting of a central vacuole, and a peripheral cytoplasm containing mitochondria, peroxisomes, nuclei (not shown) and conspicuous, lens-shaped chloroplasts with massive grana stacks (Fig. 3A). The plastids measure $5-6 \,\mu\text{m}$ in length and several micrometres in width (Fig. 3A). Their photosynthetic thylakoid membranes, the grana stacks, are unusually large and sometimes contain as many as 100 thylakoids per stack (Fig. 3B). The grana stacks are typically oriented perpendicular to the vacuole tonoplast and the druse.

All methods of observation used show one druse in each palisade parenchyma cell (Fig. 3C); rarely is there more than one druse per cell (Fig. 3D). The position of the druse within the vacuole varies depending on the individual cell, as observed in paraffin sections and leaf fractures (Figs 1D, 2J and 3C, D).

Slightly oblique paradermal leaf sections stained with PAS show that hypodermal cells have very few plastids with starch grains (Fig. 3E). The palisade parenchyma cells with their conspicuous chloroplasts have more starch grains, and the spongy parenchyma cells immediately below the palisade parenchyma have plastids with much larger and more numerous starch grains (Fig. 3E). The size of the starch grains decreases in spongy parenchyma below this region toward the lower epidermis, and they are smallest in the abaxial epidermis and its guard cells (not shown).



F1G. 2. *Peperomia obtusifolia* light (LM) and scanning (SEM) electron micrographs of portions of leaves. (A) LM paraffin paradermal section through the hypodermal–palisade parenchyma region stained with chlorozol black E and partially crossed polarizers. Three distinct regions of mounds are visible with their pit fields. Druses are evident in some cells of palisade parenchyma. (B) LM paraffin section comparable with A in bright-field mode, and stained with the PAS technique. Mound regions have distinct pit fields, and subtending palisade parenchyma shows many starch grains associated with plastids. The acidic staining method removes all crystals. (C) LM higher magnification on one interface region showing mounds with multiple pit fields. (D) SEM fracture showing the underside view of palisade parenchyma where most protoplasts are missing but membrane fragments remain at most interface regions. (E) SEM higher magnification of the interface region with a portion of cell membrane still attached. (F) SEM interface region minus cell membrane showing a pit field from the palisade parenchyma side. (G) SEM higher magnification of a pit field showing remnants of plasmodesmata. (H) SEM oblique fracture through the interface region showing a put field from the palisade parenchyma with druses. (I) SEM single exposed druse dislodged from a palisade parenchyma cell. Many facets are visible, most with obvious twin planes. (J) SEM cross-section fracture through a palisade parenchyma cell showing peripheral chloroplasts surrounding a druse in the middle of a vacuole. Scale bars: (A, B) = 50 μ m; (C, D, H) = 20 μ m; (E, J) = 10 μ m; (F) = 5 μ m; and (G, I) = 2 μ m.



FIG. 3. *Peperomia obtusifolia* light (LM), scanning (SEM) and transmission (TEM) electron micrographs of portions of leaves. (A) TEM of a single palisade parenchyma chloroplast sandwiched between the cell wall and vacuole; note the extensive stacks of grana thylakoids oriented perpendicular to the vacuole (and crystal, not shown). (B) TEM showing a granum made up of many thylakoids. (C) SEM cross-section fracture through a palisade parenchyma cell showing peripheral chloroplasts surrounding two druses in the upper part of a vacuole; chloroplasts are large. (D) SEM cross-section fracture through a palisade parenchyma cell showing peripheral chloroplasts surrounding a druse in the middle of a vacuole. (E) LM paraffin oblique section in bright-field mode, and stained with the PAS method. The section shows the lowermost hypodermal layer (upper right), interface, palisade parenchyma (middle) and portion of spongy parenchyma (lower region). There are few starch grains in the hypodermis, many small ones in palisade parenchyma and much larger ones in spongy parenchyma. Starch grains are smaller in spongy parenchyma closer to the lower (abaxial) epidermis (not shown). Scale bars: (E) = 50 μ m; (C, D) = 5 μ m; (A) = 1 μ m; and (B) = 0.5 μ m.

DISCUSSION

The pantropical, species-rich, basal-angiosperm genus Peperomia, containing about 1600 species (Samain et al., 2009), are typically small in stature, and grow mainly epiphytically, and sometimes terrestrially, often under low-intensity light in moist as well as sometimes dry conditions at lower or middle elevations, and to a lesser degree at higher elevations (Kaul, 1977). The leaves of these species vary widely in their size, colour, shape, succulence and thickness, but all appear to have a basic leaf anatomy that includes a multiple layered hypodermis (adaxial multiple epidermis) (Dahlstedt, 1900; Yuncker and Grav, 1934; Murty, 1960; Datta and Dasgupta, 1977; Kaul, 1977; Takemori et al., 2003; Souza et al., 2004) subtended by a typically single-layered palisade parenchyma (median mesophyll) that consistently contains druses composed of calcium oxalate (Schüroff, 1908; Franceschi and Horner, 1980; Kuo-Haung et al., 2007; Horner et al., 2009, 2012). Below the palisade parenchyma is the spongy parenchyma. This

anatomical arrangement is characteristic for *P. obtusifolia* leaves that are >1 mm thick, glabrous and shiny on the adaxial surface, and glabrous, dull and with stomates on the abaxial surface.

This leaf anatomy in *P. obtusifolia*, shows that 75 % of the leaf volume consists of the multiple hypodermis whereas only 4 % consists of palisade parenchyma (median mesophyll; Gibeaut and Thomson, 1989*a*, *b*). The spongy mesophyll makes up about 18 % of the leaf volume (Gibeaut and Thomson, 1989*b*). Large variations in the percentages occur between the multiple hypodermis and the spongy parenchyma in other species, while the palisade parenchyma remains about the same percentage. The palisade parenchyma is considered to be the main photosynthetic tissue in all *Peperomia* leaves in the literature and has the majority of chlorophyll. In the genus as a whole, it is the only tissue that contains the druses (Horner *et al.*, 2009, 2012) and chloroplasts with large grana

stacks (Gibeaut and Thomson, 1989*a*; Kuo-Huang *et al.*, 2007; this study).

The P. obtusifolia multiple hypodermis is basically a water-containing (Wasser Zellen: Haberlandt, 1904: Schürhoff, 1908) tissue that most authors call a 'multilayered window tissue' immediately above the photosynthetic palisade parenchyma. The present study shows the interface between these two tissues to consist of a complex, relatively thick, aggregate double wall with large, thin pit fields containing connecting plasmodesmata. These pit fields are probably mainly middle lamella, and more than likely serve as additional skylights or windows allowing light waves filtering through the multiple hypodermis to enter into the photosynthetic palisade layer. This vast photosynthetic network of cells containing multifaceted druses is able to collect and disperse the light waves to the surrounding chloroplasts with exceptionally large grana.

Kuo-Huang et al. (2007), working with P. glabella, showed that plants growing under lower light intensities had higher photosynthetic rates and larger druses than plants growing at higher light intensities. They also showed that, at lower light intensities, the vacuolar druses were larger and nearer the base of the palisade cells, whereas at higher light intensities they were smaller and near the top of the vacuoles, closer to the hypodermal-palisade parenchyma interface; and the plastids had modified thylakoids. In unpublished results (H.T. Horner), normally appearing Peperomia druses were present under lower light intensity conditions, and at high-light intensities the druses became partially dismantled and mis-shapen. Therefore, it seems from the study by Kuo-Huang et al. (2007) that light intensity has a major impact on the location, size and shape of the druses, as well as the location and ultrastructure of the chloroplasts. The presence of large stacks of grana and their perpendicular orientation to the vacuolar druses (this study) also suggest an optimization for light gathering and photosynthetic efficiency at low-light intensities. When light intensity is too high, movements of the plastids and druses seem to change in ways to protect the system against photooxidation. It is more than likely that the special pit fields in the interface region do not control the passage of light waves but have evolved to serve only to provide an efficient means for light to reach druses and the chloroplasts when conditions are right. Observing this region in other species normally growing under low- and higher light conditions should help to determine the consistency and significance, if any, of the special pit fields.

Horner *et al.* (2012), using bright-field microcinematography with fresh vibratome sections of three *Piper* species, observed the vacuole crystal sand in chlorenchyma cells actively to tumble. Similar sections of *P. obtusifolia* were viewed in the same way, but no movement of the palisade parenchyma druses was noted, even though images of fixed tissue showed druses in different locations within the vacuole, as noted by Kuo-Haung *et al.* (2007) and in this study. Horner *et al.* (2012) suggested that in vibratome cross-sections, the *Peperomia* palisade parenchyma were perpendicular to their normal orientation in the leaf and, therefore, would not move in this light path. With *Piper* crystal sand, all orientations are equal and thus any orientation could stimulate the crystals to move. In both taxa, 'movement' (as seen by different

positions in cell vacuoles) from static images and cinematography suggests that the crystals, surrounded by chloroplasts, appear to be light sensitive and thus may play a role in light collection and dispersion for photosynthesis. The mechanism for such movements is unknown.

Holthe et al. (1992) and Kuo-Huang et al. (2007) present data and interpretations of studies showing that species of Peperomia have three photosynthetic mechanisms: C₃, CAM and CAM-cycling. The latter two mechanisms are common in some families with epiphytes and they are thought to be ways to improve photosynthetic efficiency by allowing light to reach the C₃ photosynthetic tissues, and as a physiological adaptation to drought or water stress (Ting, 1985); both conditions occur in Peperomia. In their study dealing with 93 Peperomia species, Holthe et al. (1992) found that 45 (48.4%) had C₃ photosynthesis, 18 (19.4 %) had solely CAM and 30 (32.2 %) had CAM-cycling. Ting et al. (1994) used tissue printing to detect proteins and RNA associated with these photosynthetic mechanisms in the multiple hypodermis, and photosynthetic palisade and spongy parenchymas. In CAM and CAM-cycling species, CAM occurs predominantly in the multiple hypodermis and spongy parenchyma, whereas C₃ photosynthesis is primarily limited to the palisade parenchyma. In their study, Helliker and Martin (1997) considered P. obtusifolia to have CAM-cycling that includes C₃ photosynthesis.



FIG. 4. Diagram of a *Peperomia obtusifolia* palisade parenchyma cell showing the interface of the common wall with the hypodermis containing pit fields, and a protoplast with a large central vacuole containing a multifaceted druse surrounded by chloroplasts with large grana oriented perpendicular to the druse. Visible light waves are shown filtering through hypodermal pit fields, striking druse facets and dispersed to surrounding chloroplasts.

It was of interest to compare the list of 93 Peperomia species by Holthe et al. (1992) summarizing these three mechanisms with the list of 87 species with crystal macropatterns studied by Horner et al. (2009, 2012). The purpose was to determine if there was a relationship between the photosynthetic mechanism and crystal macropattern. Twenty-six species were common to both studies. The three major crystal macropatterns for these 26 species were DU, DUVbigAsmall and DR. For a photosynthetic mechanism, ten have C_3 (38.4%), eight have CAM (30.8%) and eight have CAM-cycling (30.8%). Combining DU and DUVbigAsmall macropatterns where druses uniformly occur throughout the lamina, six display C_3 , seven display CAM and seven display CAM-cycling. For the DR macropattern, four display C_3 , one displays CAM and one displays CAM-cycling. Because of the small number of species shown with the three photosynthetic mechanisms and crystal macropatterns, the summary data do not show any selective advantage for having a particular photosynthetic mechanism associated with a specific crystal macropattern. These two characters, along with their leaf anatomy, may have evolved independently but together to allow the genus as a whole to cope successfully with their specialized environments.

The combination of low-light intensity, periods of water stress, multiple hypodermis, photosynthetic palisade parenchyma containing druses and chloroplasts with large grana, and the wall interface with unusual pit fields between these two layers, provides a unique opportunity to pursue the possible function of this integrated system further (Schüroff, 1908; Franceschi and Horner, 1980; Kuo-Huang *et al.*, 2007). The present study is the first step in describing the spatial, anatomical and ultrastructural characteristics of this wall interface (Fig. 4) and provides a 'window' of opportunity to develop future studies to answer questions remaining about its unique and biologically intriguing properties.

ACKNOWLEDGEMENTS

I want to thank the Department of Genetics, Development and Cell Biology for partial support of this research, Mr Steve Mahoney, greenhouse manager of the Richard Pohl Conservatory (on top of Bessey Hall), for providing the plant material, and the Microscopy and NanoImaging Facility for research space and use of its instrumentation.

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