PERUVIAN WINDOW-LEAVED PEPEROMIA TAXA DISPLAY UNIQUE CRYSTAL MACROPATTERNS IN HIGH-ALTITUDE ENVIRONMENTS

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Editor: Bruce K. Kirchoff

Premise of research. Window-leaved *Peperomia* taxa (WPs) occur in the Peruvian Andes from near sea level to high altitudes and display curled leaves that are generally exposed to high irradiance and periodic drought, far different from the typically uncurled humid-forest, often-shaded *Peperomia* taxa. Even though representatives of the latter taxa, as well as other members of the Piperales, have been observed for the presence (type) and location (macropattern) of leaf crystals in previous studies, this special group of WP taxa has missed scrutiny. It was important to determine whether the WP taxa contained the same types of crystals in the same tissue locations and, if not, what any differences could be that are related to their anatomy, environment, and physiology.

Methodology. Living leaves of 35 available accessions from the 42 currently recognized taxa of WPs were chemically fixed, some cleared and/or vibratome sectioned or fractured to observe the internal leaf tissues for the presence of crystals composed of calcium oxalate with polarizing microscopy and SEM.

Pivotal results. WPs variably displayed three types of crystals, i.e., druses, prisms, and crystal sand, in the three major leaf tissues (multiple epidermis/hydrenchyma and palisade and spongy parenchymas). The WPs are distinctly different from uncurled humid-forest, often-shaded *Peperomia* taxa by often having prisms and crystal sand in their hydrenchyma and consistently having crystal sand in their spongy parenchyma. These results are additional synapomorphies for WPs belonging to the distinct subgenus *Fenestratae* within the genus *Peperomia*.

Conclusions. The partial or complete enclosing of the hydrenchyma by leaf curling and the resulting increased exposure of the abaxial leaf surface, with the subtending spongy parenchyma containing primarily crystal sand, suggests that these adaptations may provide protection for the primary internal photosynthetic tissue (palisade parenchyma) against photoinhibition by filtering and dispersing the solar irradiance and moderating the internal leaf temperature, two critical conditions allowing these taxa to live in extreme environments.

Keywords: calcium oxalate, crystal sand, druses, macropatterns, Peperomia, Piperaceae, Piperales, prisms, subgenus Fenestratae, window leaves.

Introduction

Inorganic calcium oxalate (CaOx· $[x]H_2O$) crystals and their aggregates occur in a variety of organisms, including lichens, the green eukaryotic algae, and vascular plants such as ferns, gymnosperms, and angiosperms (Horner et al. 2015), as well as in fungi (Arnott 1995) and mammals (Hodgkinson 1977). The question of their existence in living plants serves as the basis for understanding their physical, biochemical, and physiological properties and how these properties may serve in functional ways in the organisms that produce them (Franceschi and Horner 1980; Horner and Wagner 1995; Nakata 2003; Franceschi and Nakata 2005).

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Manuscript received April 2016; revised manuscript received August 2016; electronically published December 6, 2016.

Several functions have been suggested for plant crystals. These include calcium storage (Ilarslan et al. 2001), protection (Thurston 1976), sequestration of excess calcium and/or oxalate and heavy metals (Franceschi and Nakata 2005), an internal source of carbon dioxide (Tooulakou et al. 2016), and light gathering and reflection (Schürhoff 1908; Franceschi and Horner 1980; Kuo-Huang et al. 2007; fig. 5 and app. S1 of Horner et al. 2012).

The role of light gathering and reflection is of vital importance because of a plant's ability to exist in environments where there is either too much irradiance or barely enough irradiance to carry out photosynthesis. In the former situation, the involvement of altitude, extreme temperatures, intense irradiance, and periodic dry conditions add a variety of factors affecting a plant's response and modification to deal with these physical stresses and maintain stability within its external and internal environments (Caldwell 1968). These plants include the cacti (Hartl et al. 2007) and their relatives and members of several other angiosperm families bearing so-called window leaves, in which a hydrenchyma (nonphotosynthetic tissue consisting of large water-containing cells) serves as a front to the chlorenchymatous tissue(s). Examples of these families are Aizoaceae (*Fenestraria*. N.E.Br. [Rauh 1997]; *Lithops* N.E.Br. [Martin et al. 2013]), Commelinaceae (*Callisia navicularis* [Ortgies] D.R. Hunt [Rowley et al. 2002]), Asphodelaceae (*Haworthia* Duval), and Urticaceae (*Pilea serpyllacea* Kunth [Rauh 1959]).

In the magnoliid order Piperales, the vast majority of *Peperomia* species leaves are fully exposed, but some have a similar hydrenchymatous tissue, either between the incident irradiance and the photosynthetic chlorenchymatous tissues (Horner et al. 2015) or partially or completely enclosed by them. Prominent examples of such leaves are the species belonging to *Peperomia* subg. *Fenestratae*, commonly referred to as the window-leaved peperomias (WPs; Rauh 1971).

Peperomia subg. *Fenestratae* Pino (Frenzke et al. 2015) consists of 42 currently known succulent taxa, mainly distributed in seasonally dry tropical forest in the rain-shadowed inter-Andean valleys of northern Peru. The environment of most of these taxa is characterized by periodic and potentially extreme drought conditions and high solar irradiance. Although one taxon occurs near sea level and a few others below 1000 m, most of them grow in the high Andes, between 2000 and 3800 m, and nearly all taxa display crescent-shaped (curled) window leaves (e.g., Pino et al. 2012). The fully exposed leaves are mostly positively curled, reducing the window size (adaxial epidermis), covering the internal hydrenchyma, and exposing the photosynthetic chlorenchymatous tissues to solar irradiance through the abaxial surface either directly or by reflection from the ground.

Much work has been published to reconstruct natural relationships and to clarify and revise the systematics of Peperomia and subdivisional names within the genus. Samain et al. (2007) provided an overview of all previously published infrageneric names, including typification where required, albeit without any conclusion as to whether specific names could be applied to a modern classification reflecting molecular systematics. Initial molecular phylogenetic studies (Wanke et al. 2006, 2007; Samain et al. 2009) provided the first insights about natural relationships. Most recently, these efforts resulted in the most comprehensive circumscriptions of Peperomia subgenera, reflecting natural relationships and also strongly supporting the monophyly of Peperomia subg. Fenestratae (Frenzke et al. 2015). However, no molecular phylogenetic hypothesis on the species level is yet available for Peperomia subg. Fenestratae, preventing conclusions about crystal evolution at the species level. The WP taxa of this subgenus have previously been described with regard to their general anatomy (Rauh and Hutchison 1973; Rauh and Barthlott 1975; Kaul 1977; Christensen-Dean and Moore 1993). The presence, location, and shape of leaf calcium oxalate crystals/crystal aggregates in this subgenus, however, have eluded investigation and serve as the focus in this study.

Crystal types vary significantly within Piperales (Horner et al. 2015) and suggest certain possibilities concerning their involvement in photosynthetic efficiency. We provide here a detailed investigation of a broad and representative sampling of *Peperomia* subg. *Fenestratae* taxa. Our primary hypothesis is that leaf shape, crystal types, and the tissue location of crystals are adaptations to the environment and may be correlated with altitude. It is our contention these crystal macropatterns also may represent an ecophysiological response to photosynthetic activity, irradiance protection, and/or altitude. Therefore, this study adds information about leaf crystal types and macropatterns to the body of knowledge already published on crystals in the order Piperales (Horner et al. 2009, 2012, 2015; Horner 2012) and adds yet another facet to better understanding the potential function of crystals and their importance to plant systems in which they occur.

Material and Methods

The majority of accessions studied were collected in the wild by two of the authors (S. Wanke and M.-S. Samain) and are now growing under optimal greenhouse conditions at the Botanical Garden of Ghent University, Belgium (average minimum and maximum temperatures for 2015: 18°C/64°F and 31°C/88°F, respectively; relative humidity: between 70% and 85%). These conditions are not comparable to the droughtstress conditions that the plants seasonally experience in the wild. Although growth conditions in the greenhouse are different from those in the wild, especially with respect to seasonality, we never noticed any differences in leaf morphology between cultivated and wild plants, other than slightly increased succulence due to the less dry greenhouse environment. It should also be noted that morphological traits are diagnostic characters to distinguish between taxa (see Pino et al. 2012).

Leaves or entire stems with leaves of 31 identified *Peperomia* subg. *Fenestratae* taxa and four unidentified species (fig. 1) were removed from the plants and immersed in 70% ethanol for shipping to Ames, Iowa, for microscopic processing and analy-



Fig. 1 Phylogenetic tree showing relationship of *Peperomia* subg. *Fenestratae* (black) to other subgenera of *Peperomia* (gray). *Peperomia* subg. *Fenestratae* contains most of taxa designated window-leaved *Peperomia* taxa. Modified after figure 1 of Frenzke et al. (2015).

sis. The sampled accessions, including taxon names, collection information, and altitude (range), are provided as supporting material (appendix). If an altitude range was noted for a taxon, the range was assigned according to the herbarium label information in our digital database of herbarium specimens (2640 specimens for subg. *Fenestratae*) and additional personal observations. Specimens for which neither the altitude range nor the collection altitude was available were not included in subsequent statistical analyses.

Upon arrival in Ames, the leaves were transferred into formalinacetic acid-ethanol (FAA; Ruzin 1999) and stored in this fixative at room temperature for later processing. Several leaves were randomly chosen and hydrated to deionized water, and five to ten 275-400- μ m-thick cross sections through the middle of the leaves (slightly thicker than the largest hydrenchyma cells) were made with a vibratome (Ted Pella tpi-3000; www .tedpella.com). Some sections were mounted in water and directly observed and imaged with an Olympus SZH10 stereomicroscope (www.olympus-global.com) and an Olympus BH40 compound microscope using bright-field and polarizing optics. Alternatively, vibratome sections were placed for 1–3 h in 2.5% sodium hypochlorite (diluted 1:1 Clorox:water; www.clorox .com) until they became white and retained only cell walls and inorganic crystals. These latter sections were thoroughly water washed and then dehydrated in steps to pure ethanol, followed by 1:1 ethanol:xylenes and pure xylenes (to clear the sections; Fisher Scientific [www.fishersci.com]). Sections were subsequently placed into pure Permount (Fisher Scientific) mounting medium diluted 1:1 with xylenes and finally mounted on glass slides in pure Permount before coverslips and lead weights were added to keep sections flat and to make preparations permanent.

Both the dissecting and compound microscopes were fitted with Zeiss MRc color digital cameras (www.zeiss.de) using Zeiss AxioVision software. Images were captured in tiff format, and images were processed in both Adobe CS5 PhotoShop and Illustrator (www.adobe.com).

SEM was carried out on selected accessions to identify and verify crystal types associated with their specific leaf tissues. The FAA-fixed leaf material was transferred into 50% ethanol, further dehydrated to pure ethanol, transferred into a criticalpoint apparatus (Denton Vacuum; www.dentonvacuum.com) where ethanol was replaced with liquid carbon dioxide, and dried. The dried leaves were sliced open with clean razor blades, mounted on aluminum stubs with double-sided tape, painted at their edges with silver paint, and sputter-coated with 10 nm gold-palladium (40:60). Leaf slices were digitally imaged with a JEOL 5800 SEM (www.jeol.com) at 13 kV. Digital images were processed as described above.

Adaxial and entire-leaf epidermis lengths were determined from vibratome near-median cross sections with Zeiss Zen 2 software and the curve (spline) function. The ratio of the adaxial leaf epidermis (window) length in section and the combined length of the entire leaf epidermis in section was calculated. Spearman's rank correlation between this ratio and altitude was calculated with R 3.1.1 (R Development Core Team 2014) and RStudio 0.98.978 (RStudio 2014). A scatter plot was drawn on the altitude of the samples and the ratio, and a simple linearregression model was fitted to the data with R 3.1.1. Dot plots were drawn on the presence/absence of crystals and the crystal types of individual leaf tissues and the entire leaf, respectively, as a function of altitude or epidermis length ratio with R 3.1.1 and the R package "lattice" (Sarkar 2008).

Results

Two main features separate taxa of *Peperomia* subg. *Fenestratae* from the majority of all other *Peperomia* taxa. The first feature is the positive curling of the thick WP leaf laminas (fig. 2A–2G). The adaxial surface epidermis (window) is subtended by a large, multicellular-multilayered epidermis (hydrenchyma; Kaul 1977) that becomes partially or completely enclosed (surrounded) by the abaxial leaf surface. The latter exposes the subtending (inner) spongy parenchyma and the palisade parenchyma directly to the solar irradiance (fig. 2A [arrows]; detail of tissues in fig. 2H, 2I). The second feature is the major difference in the types of crystals/crystal aggregates that are present between the WPs and the non-WPs and, particularly, their tissue locations within the leaves.

Leaf Shape

Vibratome cross sections approximately through the middle of the leaves showed the leaf laminas to be relatively thick and variable in overall size and curling (fig. 2A-2G). The general leaf anatomy for all investigated WPs consisted of an adaxial epidermis (window; fig. 2H) followed by a large-celled, multi-cellular hydrenchyma (fig. 2H, 2I), a rather small photosynthetic palisade parenchyma (fig. 2I-2K), and a much larger spongy parenchyma and typically resides next to the palisade parenchyma (fig. 2J). The spongy parenchyma is bounded externally by a single-layered abaxial epidermis (fig. 2I).

The leaf epidermis length ratio (adaxial epidermis length/ total epidermis length) measured in near-central cross sections ranged from 0.01 to 0.47 (table 1). Leaves with a ratio closer to 0 were almost completely positively curled (closed window). Their adaxial epidermis was almost or completely absent (fig. 2E-2H), and their abaxial epidermis encircled almost the entire leaf lamina (fig. 2G, 2H), exposing its surface to the solar irradiance. Leaves with a ratio close to 0.5 exhibited a more "open window" (figs. 2A-2C), where the adaxial surface was least curled or somewhat curled.

Macropattern of Crystals/Crystal Aggregates in Leaf Tissues

All 31 identified taxa displayed one or more types of crystals/ crystal aggregates in one or more of their three major leaf tissues (fig. 3; table 1). Three crystal types were observed: druses, which are spherical crystal aggregates (fig. 3G, 3H), prisms (fig. 3E, 3F), and crystal sand consisting of small, multifaceted individual crystals (fig. 3A-3C). The distribution of crystal types per accession in the three leaf tissues and whole leaf is summarized in table 1 and figure 4. The 35 taxa displayed 15 different crystal macropatterns (table 1), some minor variations of others.

The hydrenchyma was characterized by the occurrence of prisms in most taxa (fig. 3E, 3F); some species showed both crystal sand and prisms (fig. 3A, 3D), and a few taxa contained only crystal sand (fig. 3B-3D). A few taxa revealed



Fig. 2 A-G, Stereo microscope images of vibratome near-median leaf cross sections of window-leaved *Peperomia* taxa, showing leaf curling from open (O) to partially closed (PC) to closed (C). *A*, *P. congesta*, O; AB = abaxial surface; AD = adaxial surface; H = hydrenchyma; PP = palisade parenchyma; SP = spongy parenchyma. *B*, *P. naviculifolia*, O. *C*, *P. cymbifolia*, PC. *D*, *P. ferreyrae* var. *ferreyrae*, PC. *E*, *P. dolabriformis* var. *confertifolia*, PC. *F*, *P. dolabriformis* var. *confertifolia*, PC. *F*, *P. dolabriformis* var. *confertifolia*, PC. *G*, *P. dolabriformis* var. *confertifolia*, C. *H–K*, SEM images of leaf fractures. *H*, *P. dolabriformis* var. *confertifolia*, closed apex showing lack of adaxial epidermis with abaxial epidermis surrounding outer surface of leaf. *I*, *P. dolabriformis* var. *confertifolia*, base of leaf; the large central region is hydrenchyma and toward outside palisade parenchyma, vascular bundle spongy parenchyma and abaxial epidermis. *J*, *P. dolabriformis* var. *confertifolia* at leaf base, showing large central vascular bundle in spongy parenchyma next to palisade parenchyma and large hydrenchyma cells. *K*, *P. nivalis* (PW27) showing three cells of a single-layered palisade parenchyma, each with a druse or disassembled druse giving an appearance of crystal sand. Scale bars = 2 mm (A-G), $500 \mu \text{ m} (H, I)$, $50 \mu \text{ m} (J)$, and $10 \mu \text{ m} (K)$.

Peperomia taxon	Sample no.	Epidermis length ratio ^a	Crystals		
			Hydrenchyma	Palisade parenchyma	Spongy parenchyma
P. asperula Hutchison & Rauh	PW03	.28	Prisms	Absent	Crystal sand
P. cereoides Pino & Cieza	PW34	.41	Prisms	Druses	Crystal sand
P. cereoides Pino & Cieza					
var. reducta Pino & Cieza	PW35	.32	Absent	Druses	Crystal sand, prisms
P. columella Rauh & Hutchison	PW04	.24	Absent	Crystal sand	Crystal sand
P. columnaris Hutchison ex				- ,	- ,
Pino & Klopfenstein	PW16	.37	Prisms	Druses	Crystal sand
P. congesta Kunth	PW02	.42	Crystal sand, prisms	Prisms	Crystal sand
P. cymbifolia Pino	PW12	.36	Prisms	Prisms	Crystal sand
P cymbifolia Pino var goodsteedii	1 11 12	100	1 1101110	1 1101110	oryotar sund
Pino & Cieza	PW/15	38	Prieme	Druses prisms	Crystal sand
P dolabriformis Kupth	PW/09	.58	Crystal sand prisms	Druses	Crystal sand
P. dolabriformic Kunth vor	1 w 0 >	.01	Crystal sand, prisins	Diuses	Crystal salid
1. doudonjonnis Kultur val.	DW/05	02	Current and	Damage	Current and
D dolahuifouuis Vunth von	PW03	.02	Crystal salid	Druses	Crystal sand
r. aotaorijormis Kultu var.	DW/OC	01	C 1 1	D	$C \rightarrow 1 \rightarrow 1$
confertifolia Yunck.	PW06	.01	Crystal sand	Druses	Crystal sand
P. dolabriformis Kunth var.	DW/4 O	0.5		D	
confertifolia Yunck.	PW10	.05	Crystal sand, prisms	Druses	Crystal sand
P. dolabriformis Kunth var.				_	
lombardii Pino	PW07	.05	Absent	Druses	Absent
P. dolabriformis Kunth var.					
<i>multicaulis</i> Pino & Cieza	PW08	.01	Crystal sand	Druses	Crystal sand
P. dolabriformis var. grandis Hutchison					
ex Pino & Klopfenstein	PW11	.04	Crystal sand	Druses	Crystal sand
P. erosa Hutchison ex Pino	PW18	.47	Absent	Druses	Absent
P. ferreyrae Yunck. var. ferreyrae	PW28	.17	Prisms	Druses	Crystal sand, prisms
P. ferreyrae Yunck.					
var. musifolia Pino	PW29	.12	Prisms	Druses	Crystal sand
P. hutchisonii Yunck.	PW19	.10	Prisms	Druses, prisms	Absent
P. liclicensis Pino & Klopfenstein	PW22	.36	Crystal sand, prisms	Druses	Crystal sand, prisms
P. maijeri Pino & Samain	PW24	.31	Prisms	Druses	Crystal sand
P. mathieui Pino & Samain	PW17	.36	Crystal sand, prisms	Druses	Crystal sand, prisms
P naviculifolia Trel	PW/01	38	Prisms	Crystal sand	Crystal sand
P nivalis Mia	PW/27	35	Crystal sand prisms	Druses	Crystal sand prisms
P minalis Mia	PW/30	.55	Crystal sand, prisms	Druses	Crystal sand, prisms
P minulia Mia f diminuta Dipo	DW/14	.27	Drieme	Druses	Crystal sand, prisins
P. minutis Miq. 1. annunuta Thio	1 W 17	.50	1 1 151115	Diuses	Crystal salid
Trol or Ding	DW/22	22	Duiomo	Current and	Current and
	PW32	.23	PTISIIIS	Crystal sand	Crystal sand
P. nivalis Miq. var. sanmarcensis	DW/21	20	A.1	D	
Pino & Cieza	PW31	.38	Absent	Druses	Crystal sand
P. samainiae Pino	PW33	.32	Prisms	Druses	Crystal sand
P. strawn Hutchison ex				_	
Pino & Klopfenstein	PW13	.36	Prisms	Druses	Crystal sand
P. wolfgang-krahnii Rauh	PW20	.25	Prisms	Druses	Crystal sand
Peperomia species 1	PW23	.33	Crystal sand	Druses	Crystal sand
Peperomia species 2	PW26	.22	Prisms	Druses	Crystal sand, prisms
Peperomia species 3	PW25	.14	Prisms	Druses	Crystal sand
Peperomia species 4	PW21	.41	Prisms	Druses	Crystal sand

Crystal Patterns of Leaf Tissues and Window Size as Ratio of Adaxial Epidermis Length to Total Epidermis Length in Window-Leaved Peperomia Taxa

Table 1

^a Adaxial leaf epidermis length/total epidermis length.

no crystals in the hydrenchyma (table 1). The palisade parenchyma was characterized by the prevalence of druses (figs. 2K, 3G, 3H) in the identified taxa. Only two taxa showed prisms in addition to druses, and a few showed solely prisms (fig. 3K) or crystal sand (fig. 3I, 3J). One taxon did not display crystals in the palisade parenchyma. Except for three taxa, which lacked crystals in the spongy parenchyma, all investigated taxa showed crystal sand in this tissue (table 1; fig. 3L, 3P), and a few taxa displayed additional prisms (fig. 3M–3O). There were no taxa found that lacked crystals in the entire leaf. Taxa showed various combinations of the three crystal types in the different tissues, but no taxon had all three types of crystal in the same tissue (table 1; fig. 4). No association between crystal types and altitude distribution (fig. 4A) or the leaf epidermis ratio (fig. 4B)



Fig. 3 Polarized light microscope (POL) images from vibratome sections and SEM images from leaf fractures showing three crystal types druses, prisms, and crystal sand—found in hydrenchyma, palisade parenchyma, and/or spongy parenchyma of selected window *Peperomia* taxa. *A*, POL: *P. nivalis* (PW27), hydrenchyma crystal sand and prisms. *B*, SEM: *P. dolabriformis* var. *confertifolia*, hydrenchyma crystal sand. *C*, SEM: *P. nivalis* (PW27), hydrenchyma crystal sand. *D*, SEM: *Peperomia* species 4, hydrenchyma crystal sand; *E*, POL: *Peperomia* species 4, hydrenchyma prisms. *F*, SEM: *P. nivalis* (PW27), hydrenchyma prisms. *G*, POL: *Peperomia* species 2, palisade parenchyma druse layer. *H*, SEM: *P. dolabriformis* var. *confertifolia*, palisade parenchyma druses. *I*, POL: *P. nivalis* var. *lepadiphylla*, palisade parenchyma crystal sand layer. *J*, SEM: *P. nivalis* (PW27), palisade parenchyma crystal sand. *K*, POL: *P. congesta*, palisade parenchyma prisms. *L*, POL: *P. nivalis* var. *lepadiphylla*, spongy parenchyma prisms. *O*, SEM: *P. nivalis* (PW27) spongy parenchyma prisms. *P*, SEM: *P. mathieui*, spongy parenchyma crystal sand. Scale bars = 50 µm (*B*, *G*, *I*, *L*, *N*), 20 µm (*D*, *K*), 10 µm (*A*, *E*, *F*, *J*, *P*), and 5 µm (*C*, *H*, *M*, O).



Fig. 4 Crystal types (prisms, crystal sand, druses) and their distribution in three leaf tissues: hydrenchyma, palisade parenchyma, and spongy parenchyma of 31 taxa (circles) of window-leaved *Peperomia* taxa in relation to (*A*) altitude (m) and (*B*) leaf epidermis length ratio (see "Material and Methods" for explanation). Table 1 specifically identifies these three characters by taxon. P = prisms; D = druses; CS = crystal sand.

was found independently from the tissue, even if the entire leaf was considered.

Although we did not observe any association between the crystal types and altitude, we found that leaf epidermis length ratio and altitude were correlated (rho = 0.545, P = .002; fig. 5). Taxa with small windows were found at lower altitudes and taxa with larger windows at higher altitudes (fig. 4*B*, *left*).

Adaxial and/or Abaxial Leaf Cuticles/Walls

When observed with polarized light, 26 of 35 investigated taxa displayed relatively thick abaxial and/or adaxial cuticles and outer epidermal walls. Sixteen of them displayed both thick abaxial and adaxial cuticles and outer epidermal walls; eight taxa displayed just thick abaxial cuticles and outer epidermal walls; and only two taxa displayed thick adaxial cuticles and outer epidermal walls.

Discussion

The overall results of this study are a major contrast in leaf curling, altitude, crystal type, and crystal macropattern between the WPs in *Peperomia* subg. *Fenestratae* and representatives of the other subgenera of *Peperomia* studied from low-altitude and often shady locations. The non-WP species display three types of crystals: druses, which are in the palisade parenchyma only; and raphide bundles or prisms sometimes occurred in the spongy parenchyma (see Horner et al. 2009, 2012; Horner 2012). The presence of crystals in the WP hydrenchyma and that of crystal sand in all three tissues (table 1), especially the spongy parenchyma, clearly separates taxa of *Peperomia* subg. *Fenestratae* from the other *Peperomia* lineages. The crystal sand, in particular, is a synapomorphy for *Peperomia* subg. *Fenestratae*.

The positive curling of the succulent WP leaves that partially or completely encapsulate the large water-containing hydrenchyma, creating a smaller adaxial epidermal window, may be an adaptation to meet environmental stresses. Jürgens (1985) alluded to leaf curling in his study of the convergent evolution of succulent leaves in certain plant families (including Piperaceae). He mentioned P. obtusifolia, a non-WP, and P. columella and P. dolabriformis, both WPs included in this study. He accurately diagrammed (his fig. 10 II) leaf curling similar to what we have shown in figure 2A-2G. We do not have an explanation for the curling of the WP leaves, but this condition could be interpreted as an evolutionary response to protecting the water capacity of leaves during drought conditions by surrounding the hydrenchyma with chlorenchymatous tissues, by an abaxial epidermis with thickened outer walls and cuticle, and by changing their surface orientation to the lowest irradiance acceptance angle.

Fifteen crystal macropatterns based on crystal type and tissue location were described in this study for the 35 WP taxa (table 1). This large number of crystal macropatterns, some just minor variations of each other, and the taxa's varying leaf



Fig. 5 Dot plot of 31 window-leaved *Peperomia* taxa (circles) showing ratio of adaxial leaf surface length to total (adaxial + abaxial) leaf surface length. Linear-regression line and regression equation are shown. Prediction between altitude and ratio is significant (P < .05); the precision of the prediction is not very good.

curling are problematic because there is no phylogenetic hypothesis of this subgenus at the present time. Based on our three previous studies of crystal macropatterns in the genus *Peperomia* and other members of the Piperales, the following three questions provide insight into our morphological and anatomical results.

Which macropattern(s) represent(s) the basic pattern in this subgenus from which the others are derived? Both druses and crystal sand were reconstructed as the ancestral state both for the most recent common ancestor of Piper and Peperomia and for Piperales as a whole (Horner et al. 2015). However, in the genus Peperomia, druses represent the dominant and ancestral crystal type in the palisade parenchyma (Horner et al. 2009, 2015), whereas crystal sand is dominant and ancestral in Piper (Horner et al. 2015). The findings in this study show that the WP taxa have both druses and crystal sand, indicating, most likely, a shift in crystal type from the ancestral druses back to crystal sand before the diversification of Peperomia subg. Fenestratae. This will require confirmation by mapping these states on the molecular phylogeny of this clade, once it becomes available. This shift of crystal type may be environmentally driven by the demands of altitude and increased irradiance.

What impacts, if any, do the environmental conditions have on leaf structure and ultimately on the crystal macropatterns observed? The 35 taxa are endemic to Peru and grow at relatively high altitudes under arid or semiarid conditions in direct solar irradiance (Frenzke et al. 2015). Our results showed that the degree of leaf curl based on altitude was the opposite of what we expected; the taxa with more-curled leaves were found at lower altitudes. These succulents are composed of hydrenchyma and the chlorenchyma (palisade and spongy parenchymas), and all five tissues, including the two epidermises, are important to successfully persisting in drought conditions and maintaining photosynthesis. In the majority of the 35 taxa, the positive curling of the leaves exposes more of the abaxial surface and the internal chlorenchyma to incident and reflected irradiance. This irradiance initially penetrates the typically thicker abaxial outer wall and cuticle, then the spongy parenchyma, and finally the palisade parenchyma. This tissue arrangement is consistent in all of the taxa studied and involves selective irradiance absorption and reflection (UV, visible, and infrared) and internal scattering. Egbert et al. (2008) studied six succulents, two of them WPs, using an omnidirectional fiber optic microprobe and covering the leaf windows to determine internal irradiance quality and intensity. They found the irradiance in different regions to be of nearly the same intensity or more intense than the surface incident irradiance. Martin et al. (2013), using an infrared thermometer to measure leaf temperature in Lithops (a subterranean leaf with only the window exposed), showed that leaf temperature varied between covered and uncovered windows. Their conclusions indicated that windows of Lithops growing under cloudy conditions were larger than windows of plants growing in less cloudy regions. This means that the size of the window is inversely correlated with irradiance that minimizes photoinhibition. These results indicate that there is abundant scattering and concentration of the absorbed irradiance by the tissues. However, these two studies did not take into account the presence and possible involvement of leaf crystals.

In an earlier study, Eller et al. (1983) found several non-WP succulent plants in the Namib Desert whose absorption values for visible global radiation did not differ from each other or from those of mesophytic plants. They did detect an increase in the infrared range with increasing succulence. Likewise, Sinclair and Thomas (1970) showed that reflectivity of leaves in arid regions was often no higher than that of leaves in mesic environments. These studies, and others cited therein, raise the issue of how irradiance (UV, visible, and infrared) entering leaves is being handled to prevent photoinhibition but sustain an irradiance intensity and internal temperature conducive to reducing stress and potentially driving evolution. The next question may provide an answer.

Do the types of leaf crystals and their tissue crystal macropatterns represent an ecophysiological response to photosynthetic activity and/or protection? Thirty-two of the 35 WPs (table 1; appendix) have crystal sand, and five of these have prisms associated with crystal sand in their spongy parenchyma. Our results showed that, contrary to our second expectation, the number and types of leaf crystals and their tissue locations showed no significant difference based on altitude. This suggests that crystals are needed for another reason, possibly as selective internal dispersers of irradiance and for temperature control.

Gal et al. (2012), in a study on the function of biominerals in leaves, showed that in-leaf and isolated inclusions of calcium oxalate (in *Carya illinoinensis*) and calcium carbonate (in *Ficus elastica*) served the function of scattering irradiance. They identified the locations and sizes of the biominerals in the leaves, using MicroCT and modulated microfluorometry to assess their irradiance-scattering properties. Their results clearly showed that both biominerals scattered irradiance effectively and that their spatial locations in the leaves provided uniform distribution of dispersed irradiance. Both biominerals were noted to have "pointed protrusions and angular surfaces, suggesting high scattering capability" (Gal et al. 2012, p. OP80). They further noted that scattering of irradiance by in-leaf biominerals is "roughly independent" of the direction of the irradiating source, meaning, in the case of the WPs, that it could be direct solar irradiance or multidirectional reflection from surrounding terrain. Conversely, in the case of the druses, crystal sand, and prisms in the three tissues of the WPs, their function could also be differential internal reflection or dispersion of irradiance to optimize photosynthesis, reduce photoinhibition, and maintain internal leaf temperature. This suggests that whatever irradiance penetrates into the leaf comes in contact with at least crystal sand. As shown in Horner et al. (2012, fig. 30 and cinematography appendix S1), when a collimated visible irradiance beam was passed through living chlorenchyma containing crystal sand of Piper nigrum, the crystal sand actively tumbled in the large, central vacuoles. This active movement suggests (similar to Gal et al. 2012) that the many individual crystals, each with multiple facets, could be dispersing the irradiance in many directions, thus reducing any focused intensity internally and therefore protecting plastids within the palisade parenchyma from photoinhibition. The situation may be the same for the infrared irradiance: dispersion to reduce heat to maintain a physiologically balanced internal temperature. These interpretations are supported by the results of Sinclair and Thomas (1970) and Eller et al. (1983). It should be further noted that 31 WPs have prisms and/or crystal sand in their hydrenchyma, again suggesting that both the spongy parenchyma and the hydrenchyma may work together in moderating incident and reflected irradiance entering leaves.

These studies are reinforced by a large number of observational and experimental works dealing with the effect of UV irradiance on a variety of plant species growing at different latitudes and altitudes. UV-B irradiance has been acknowledged as having major deleterious affects on all kinds of organisms. Caldwell (1968) provided an excellent in-depth view of how plants exposed to UV radiation are affected and some of the mechanisms plants have evolved to adapt to it (Caldwell 1968; Caldwell et al. 1982, 1983; Alexandris et al. 1999; Dvorkin and Steinberger 1999). The UV radiation that reaches the plant surfaces (adaxial and abaxial epidermises) has been tested for penetration into the leaves of a variety of plants. The general consensus is that the leaf epidermal layer in high-altitude plants may serve as the initial barrier by having a thickened outer wall and a waxy cuticle, absorbers of UV radiation (Cline and Salisbury 1966; Robberecht and Caldwell 1978; Robberecht et al. 1980; Turunen and Latola 2005). Cline and Salisbury (1966) indicated that vertically oriented leaves could also affect the degree of irradiance. Comparing this information to that for WPs, it is clear from our study that WP leaves display some degree of thickening of their epidermal outer walls and that leaf curling may adjust the angle of irradiance.

All of the factors mentioned above may be important in the evolution of the WPs to exist in new and potentially more stressful habitats at higher altitudes. However, none of these studies (except Gal et al. 2012 and this study) mentioned whether crystals occur in any of the leaf tissues that were studied. The results of our study provide a new and possibly intriguing possibility-the presence of primarily crystal sand in the hydrenchyma, but particularly in the spongy mesophyll, may play a major role in preventing the high irradiance from penetrating into the palisade parenchyma and causing photoinhibition. The humid-forest, partially shaded Peperomia taxa generally lack crystals in these two tissues and never display crystal sand. The presence and location of crystals may be a significant reason for the success of WP taxa in maintaining a physiologically balanced internal environment, allowing them to move into new niches with potentially stressful conditions.

Acknowledgments

We thank the Botanical Garden of Ghent University, Belgium, for providing greenhouse space (M.-S. Samain) and the Iowa State University (ISU) Microscopy and NanoImaging Facility for processing and imaging the WP leaves (H. T. Horner), to Sarah Chicchelly for her assistance during an ISU undergraduate Honors 200H project, to Chantal Dugardin for help with collecting and sending of material, as well as providing information on greenhouse conditions, and to Guillermo Pino for help with the altitude ranges of the WP species and sharing his profound knowledge of this group. We further thank the authorities of Peru (permits 009-2009-AG-DGFFSDGEFFS, 004-2011-SERNANPDGANP-JEF, 0271-2011-AG-DGFFS-DGEFFS, 003-2012-SERNANPJEF, and 0395-2013-MINAGRI-DGFFS/ DGEFFS) for permission to collect plant material. This study has been supported by German Science Foundation (DFG) research projects DFG NE681/5-1, DFG NE681/5-2, and DFG NE681/11-1; Research Foundation Flanders (FWO-Vlaanderen) research project FWO G.0172.07; FWO travel grants to M.-S. Samain; and the Research Group Spermatophytes, Ghent University (Belgium).

Appendix

Collection Details and Altitude Ranges of Window-Leaved Peperomia Taxa

Sampled accessions including authority, collection information, and altitude (range) are presented as follows: taxon; sample number; Botanical Garden Gent number; collection number; origin; GPS coordinates and altitude; altitude range.

P. asperula Hutchison & Rauh; PW03; 2004 1996; Guido Mathieu 181; unknown; unknown; 700-2000 m.

P. cereoides Pino & Cieza; PW34; 2009 0598; Mathieu et al. 2009–152; Peru, Cajamarca, San Marcos-Liclic; 7°22′35.1″S, 78° 03′22.2″W, 3039 m; 3000–3100 m.

P. cereoides Pino & Cieza var. *reducta* Pino & Cieza; PW35; 2010 2680; Samain et al. 2010–128; Peru, Cajamarca, road to Pampa Alegre, km 1.5; 7°13′28.0″S, 78°01′34.3″W, 3656 m; 3600–3800 m.

P. columella Rauh & Hutchison; PW04; 2007 0812; Pino 1195; Robert Maijer, private collection; unknown; 1300-1600 m.

P. columnaris Hutchison ex Pino & Klopfenstein; PW16; 2011 0056; Samain et al. 2010–227; Peru, Amazonas, dist. Chachapoyas, slope east of Río Utcubamba on road to Caclic, km 498; 13 km below and west of Chachapoyas; 6°13′27.2″S, 77°54′40.8″W, 1793 m; 1700–2200 m.

P. congesta Kunth; PW02; 2006 1012; --; Heidelberg BG 141626; unknown; 1800 m.

P. cymbifolia Pino; PW12; 2010 2710; Samain et al. 2010–163; Peru, Cajamarca, Chota-La Palma, km 5.6; 6°31′53.1″S, 78° 37′47.8″W, 2630 m; 1800–2800 m.

P. cymbifolia Pino var. *goodspeedii* Pino & Cieza; PW15; 2011 0075; Samain et al. 2010–250; Peru, Cajamarca, road Celendín-Cajamarca, km 8; 6°54′45.5″S, 78°10′49.2″W, 2940 m; 2700–3400 m.

P. dolabriformis Kunth; PW09; 2010 2700; Samain et al. 2010–150; Peru, Cajamarca, San Pablo-Chilete, km 25; 7°12′54.7″S, 78°50′12.4″W, 872 m; 850–1900 m.

P. dolabriformis Kunth var. confertifolia Yunck.; PW05; 2011 0038; Samain et al. 2010–206; Peru, Amazonas, Bagua Grande-Chachapoyas, km 272; 5°55'12.3"S, 78°07'21.8"W, 804 m; 350–810 m.

P. dolabriformis Kunth var. *confertifolia* Yunck.; PW06; 2011 0024; Samain et al. 2010–190; Peru, Cajamarca, road Jaén-Bagua Grande, km 191–192; 5°46′58.7″S, 78°42′10.2″W, 439 m; 350–800 m.

P. dolabriformis Kunth var. *confertifolia* Yunck.; PW10; 2009 0512; Mathieu et al. 2009–061; Peru, Amazonas, Pongo de Rentema; 5°29'34.8"S, 78°33'00.0"W, 377 m; 350–800 m.

P. dolabriformis Kunth var. *lombardii* Pino; PW07; 2011 0020; Samain et al. 2010–186; Peru, Cajamarca, Jaén-Chiclayo, km 135; 6°01'17.6"S, 78°59'33.6"W, 780 m; 60–800 m.

P. dolabriformis Kunth var. *multicaulis* Pino & Cieza; PW08; 2010 2675; Samain et al. 2010–122; Peru, Cajamarca, Cajamarca-La Grama, km 82; 7°25′45.6″S, 78°06′59.1″W, 2248 m; 2000–2300 m.

P. dolabriformis var. grandis Hutchison ex Pino & Klopfenstein; PW11; 2007 0832; Pino 1492; Peru; unknown; 1100-2100 m.

P. erosa Hutchison ex Pino; PW18; 2011 0043; Samain et al. 2010–212; Peru, Amazonas, road Pedro Ruiz-Chachapoyas, km 1; 6°57′13.5″S, 77°58′41.5″W, 1333 m; 1200–1400 m.

P. ferreyrae Yunck. var. *ferreyrae*; PW28; 2011 0066; Samain et al. 2010–238; Peru, Amazonas, Limon Punta-Leymebamba, km 56.3; 6°37′23.0″S, 77°48′49.4″W, 2022 m; 1400–2050 m.

P. ferreyrae Yunck. var. musifolia Pino; PW29; 2011 0054; Samain et al. 2010–225; Peru, Amazonas, road Chachapoyas-Rodríguez de Mendoza, km 2.9; 6°13'31.1"S, 77°51'26.6"W, 2277 m; 2250–2400 m.

P. hutchisonii Yunck.; PW19; 2007 0768; Pino 833; Peru; unknown; 400-700 m.

P. liclicensis Pino & Klopfenstein; PW22; 2007 0830; Pino 694; Peru, Cajamarca, near Liclic, path Totorilla-El Platanillo; 7°19'52.1"S, 77°59'35.4"W, 2991 m; 2900–3100 m.

P. maijeri Pino & Samain; PW24; 2010 1024; Samain et al. 2010–062; Peru, Ancash, prov. Huari, town of Masín; 9°21′55.0″S, 77°05′27.4″W, 2579 m; 2500–3400 m.

P. mathieui Pino & Samain; PW17; 2011 0050; Samain et al. 2010–221; Peru, Amazonas, Pedro Ruiz-Chachapoyas, km 15; 6°03'31.2"S, 77°55'09.8"W, 1458 m; 1400–2200 m.

P. naviculifolia Trel.; PW01; 2006 1010; --; Heidelberg BG 141619; unknown; 2500-2900 m.

P. nivalis Miq.; PW27; 2007 0801; ---; Peru, Huánuco, Tomayquicha; unknown; 2600-3200 m.

P. nivalis Miq.; PW30; 2007 0797; Pino 917; Peru; unknown; 2600-3200 m.

P. nivalis Miq. f. *diminuta* Pino; PW14; 2011 0076; Samain et al. 2010–251; Peru, Cajamarca, Celendín-Cajamarca, km 60.2; 7°04′53.4″S, 78°20′05.5″W, 3187 m; 2600–3200 m.

P. nivalis Miq. var. *lepadiphylla* Trel. ex Pino; PW32; 2009 0710; Samain et al. 2009–111; Peru, Cuzco, near the ruins of Ollantaytambo; 13°15′27.4″S, 72°16′03.5″W, 2910 m; 2200–3500 m.

P. nivalis Miq. var. *sanmarcensis* Pino & Cieza; PW31; 2009 0601; Mathieu et al. 2009156; Peru, Cajamarca, road from San Marcos to Cajamarca, track "Huayobamba"; 7°18′53.8″S, 78°11′11.0″W, 2390 m; 2200–2700 m.

P. samainiae Pino; PW33; 2009 0789; Samain et al. 2009–206; Peru, Junín, along the road Acobamba-Palcamayo, km 1; 11°20′55.3″S, 75°39′35.7″W, 2980 m; 2900–3200 m.

Peperomia species 1; PW23; 2007 0828; Pino 1193; Peru; unknown; unknown.

Peperomia species 2; PW26; 2003 1369; Pino 708; -; unknown; unknown.

Peperomia species 3; PW25; 2003 1337; -; Peru; unknown; unknown.

Peperomia species 4; PW21; 2007 0827; Werner Rauh 2029; Peru; unknown; unknown.

P. strawii Hutchison ex Pino & Klopfenstein; PW13; 2010 2686; Samain et al. 2010–134; Peru, Cajamarca, prov. San Marcos, dist. Pedro Galvez, Catagón; 7°20'04.2"S, 78°11'44.9"W, 2241 m; 1500–2300 m.

P. wolfgang-krahnii Rauh; PW20; 2001 2341; Heidelberg BG sn; Peru; unknown; 1600 m.

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