Uncovering the mechanisms of novel foliar variegation patterns caused by structures and pigments

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ABSTRACT: In nature, foliar variegation has varied origins, and can be ascribed to two major mechanisms: pigment-related variegation and structural variegation caused by the optical properties of leaf structure. However, understanding of these mechanisms is still lacking, and structural mechanisms are often misinterpreted. In this study, six variegated plants native to Taiwan and two ornamental plants, with novel and unusual foliar variegation patterns, are studied to reveal their mechanisms of variegation. Two newly understood variegation patterns, the silvery white leaf surface of Begonia aptera and the varnish on basal leaflets of Oxalis corymbosa, are reported here. White to light green patches on leaf surfaces characterize the foliar variegation in the other six study species, Nervilia nipponica, O. acetosella subsp. griffithii var. formosana, Paphiopedilum concolor, Selaginella picta, Smilax bracteata subsp. verruculosa and Valeriana hsiui. All six Taiwan native plants exhibit structural variegation, five of which have air space type variegation. Surprisingly the silvery white leaf surface of B. aptera results from numerous sand-like white spots caused by intercellular space. The varnish on leaves of O. corymbosa is the epidermis type of variegation comprised of two newly identified subtypes, larger epidermal cells and thicker outer cell walls. The two ornamental plants have variegation caused by pigments: the chloroplast type from fewer chloroplasts in P. concolor and the chlorophyll type, from absence of chloroplasts in S. picta. This study extends understanding of the mechanisms of natural foliar variegation, illustrating the diversity of mechanisms by which plants may change the appearance of their leaves.

KEY WORDS: chlorophyll type, chloroplast type, epidermis type, pigment-related variegation, structural variegation.

INTRODUCTION

Variegated plants are popular in flower markets and gardens. Although they occasionally occur in nature, variegated plants are relatively commonly found in shaded habitats and forest understories (Givnish, 1990). Variegation is defined by the presence of multiple colors on leaf surfaces displaying regular or irregular patterns. Interestingly, some plants are found to have variegated leaves only in the juvenile stage (e. g. Blastos cochinchinesis Lour., Melastomataceae) (Chen et al., 2017), but some plants remain variegated leaves throughout life (e. g. Begonia formosana [Hayata] Masam., Begoniaceae) (Sheue et al., 2012). The adaptive significance of foliar variegation is not fully understood (Tsukaya et al., 2004). However, foliar variegation has been reported to be associated with genetic mutations (Sakamoto et al., 2009), environmental factors (Burger et al., 1988), photoprotection (Esteban et al., 2008) and reducing herbivore damage (Campitelli et al., 2008).

Based on a study of 55 variegated plants from 24 families, Hara (1957) identified four mechanisms of foliar variegation: pigment type, chlorophyll type, epidermis type and air space type. However, new mechanisms of variegation have been reported recently. For example, the crystal type and chloroplast type have been found in B. cochinchinensis in white areas on a leaf, which are created by additional calcium oxalate crystals in the adaxial epidermal cells, and colorless spongy-like upper mesophyll cells with smaller and fewer chloroplasts (Chen et al., 2017).

The variegation mechanisms mentioned above fall into two groups: pigment-related variegation (chemical color, caused by pigments) and structural variegation (physical color, caused by the optical properties of leaf structure) depending on how light areas of a leaf are created. Although physical color is easily observed in many animals, few cases of physical color are well known from plants (Gould and Lee, 1996; Pao et al., 2018). Nevertheless, there have been recent reports of unusual structures creating foliar variegation patterns in plants (Fooshee and Henny, 1990; Tsukaya et al., 2004; Konoplyova et al., 2008; La Rocca et al., 2011; Sheue et al., 2012; Zheng et al., 2016; Chen et al., 2017). From these reports, it appears that variegation patterns caused by diffuse reflection of light from intercellular space are a common case of physical color in plants. Little or no loss of photosynthetic function, with variegation caused by physical color (air space type), has been documented in Begonia leaves (Sheue et al., 2012) based on chlorophyll fluorescence parameters (Fv/Fm, maximum quantum yield of PSII). In contrast, variegation caused by chlorophyll
Table 1. Plant materials of eight taxa, with variegation pattern, mechanism of foliar variegation, collected location and voucher specimen.

<table>
<thead>
<tr>
<th>taxon (Family)</th>
<th>variegation pattern</th>
<th>mechanism of foliar variegation</th>
<th>collected location</th>
<th>number of individuals</th>
<th>number of leaves</th>
<th>voucher specimen**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Begonia aptera Blume (Begoniaceae)</td>
<td>silvery white (close view: sand-like spots)</td>
<td>air space type</td>
<td>1</td>
<td>3</td>
<td>9</td>
<td>Pao 5</td>
</tr>
<tr>
<td>Nervilia nipponica Makino (Orchidaceae)</td>
<td>white, patches</td>
<td>light green, patches</td>
<td>air space type</td>
<td>chloroplast type</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Paphiopedilum concolor Pfitzer* (Orchidaceae)</td>
<td>white, advein</td>
<td>air space type</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>Pao 6</td>
</tr>
<tr>
<td>Oxalis acetosella subsp. griffithii (Edgew. &amp; Hook. f.) Hara var. formosana (Terao) Huang &amp; Huang (Oxalidaceae)</td>
<td>varnish, patches</td>
<td>epidermis type (cell size, cell wall thickness)</td>
<td>4</td>
<td>3</td>
<td>9</td>
<td>Pao 8</td>
</tr>
<tr>
<td>Oxalis corymbosa DC. (Oxalidaceae)</td>
<td>white, patch (apex of the middle leaf)</td>
<td>chlorophyll type</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>Liu JW 13</td>
</tr>
<tr>
<td>Selaginella picta (Griff.) A.Br. ex Baker* (Selaginellaceae)</td>
<td>light green, patches</td>
<td>air space type</td>
<td>7</td>
<td>3</td>
<td>9</td>
<td>Pao 10</td>
</tr>
<tr>
<td>Smilax bracteata C. Presl subsp. verruculosa (Merr.) Koyama (Smilacaceae)</td>
<td>patches</td>
<td>air space type</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>Pao 7</td>
</tr>
</tbody>
</table>

1. Huisun Forest of National Chung Hsing University in Nantou, Taiwan. 2. Baxianshan National Forest in Taichung, Taiwan. 3. Cinsbu forest in Hsinchu, Taiwan. 4. National Chung Hsing University in Taichung, Taiwan. 5, A flower market in Taichung, Taiwan. 6, Dr. Cecilia Koo Botanic Conservation Center in Pingtung, Taiwan. 7, Lienhuachih forest in Nantou, Taiwan. *Alien ornamental plant.; **All Specimen were deposited in TCB herbarium.

deficiency (Bae et al., 2000) may lead to significantly reduced performance of photosynthesis with cost to the plants (Zeliou et al., 2009; Sheue et al., 2012).

Taiwan is a mountainous island with a high diversity of vascular plants. Over 4000 species of vascular plants are well documented (Hsieh and Shen, 1993). Some species exhibit foliar variegation in nature (Chen et al., 2017; Pao and Sheue, personal observation). However, information on variegation is mostly lacking in plant floras. In this study, we select eight variegated plants, including six native plants and two ornamental plants, which display unusual foliar variegation patterns rarely seen in other plants. These include Begonia aptera Blume (Begoniaceae) with silvery white leaves, Nervilia nipponica Makino (Orchidaceae) with silvery white patches on the leaves, and Oxalis corymbosa DC. (Oxalidaceae) with varnish marks. Using anatomical techniques, this study uncovers the variegation mechanisms behind these unusual and remarkable patterns, revealing new mechanisms of foliar variegation and furthering understanding of variegation mechanisms.

MATERIALS AND METHODS

Plant materials

We studied variegated leaves of eight taxa from six families (Table 1). Six of these taxa are native to Taiwan growing in shade environments under forest canopies, ranging from low land [B. aptera, O. corymbosa. and Smilax bracteata C. Presl subsp. verruculosa (Merr.) Koyama] (Fig. 1A, J; Fig. 2G) to middle elevation [N. nipponica, O. acetosella subsp. griffithii var. formosana and Valeriana hsuii] (Fig. 1D, G; Fig. 2I). Based on our field observations, at least three taxa (B. aptera, O. acetosella subsp. griffithii var. formosana and O. corymbosa) show leaf color polymorphisms, with both variegated and normal green (non-variegated) individuals in the same populations. Two taxa (S. bracteata subsp. verruculosa, V. hsuii) show strong variegation traits at early stages, although variegation may not be evident in later leaves. However, N. nipponica shows foliar variegation in all leaves. Two ornamental plants with unusual variegated patterns are also included, Paphiopedilum concolor Pfitzer (Orchidaceae) and Selaginella picta (Griff.) A.Br. ex Baker (Selaginellaceae) (Fig. 2A, D). Voucher specimens were deposited in the Herbarium of National Chung Hsing University (TCB) in Taichung, Taiwan.

Leaf structure

Two to three fresh leaves were collected from each of two to three individuals of each taxon. For all leaf samples, small pieces (1.0–2.0 × 1.0–2.0 mm²) were cut from both green and light areas were cut. Using a general electron microscopy protocol (Sheue et al., 2012), these pieces were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) overnight at 4°C, subject to dehydration, embedding, and cutting with a microtome to obtain semithin (1 μm thick) sections (Kuo-Huang and Chen, 1999). All sections were observed with a light microscope (Olympus, BH-2, Tokyo, Japan).

Comparative features between green and light areas

For further understanding of the variegation mechanisms, the variegated leaves of two variegated plants, O. corymbosa and P. concolor, were compared. Five individuals of O. corymbosa were studied and compared. One mature leaf with evident variegation pattern was chosen from each individual. Five values of cell wall thickness were averaged for both the green and light areas on the same leaflet. To compare adaxial epidermal cell size, samples (c. 1 × 1 cm²) were placed on a cold stage (prefrozen by liquid nitrogen) for 1–2 min, and observed with a tabletop scanning electron
Fig. 1. Variegated plants with silvery white (A), white (D, G) and varnish (J) patterns on adaxial leaf surfaces and comparative leaf structures between green and light areas. (A–C) Begonia aptera. (A) Two individuals in the field, a green one on the left and a silvery white one on the right. Insets are higher magnification images of leaf surfaces of both individuals. (B–C) Leaf structures in a green area in (B) and a light area in (C). (D–F) Nervilia nipponica. (D) A leaf with irregular patches almost covering the entire leaf. (E–F) Leaf structures in a green area in (E) and a light area in (F). (G–I) Oxalis acerosella subsp. griffithii var. formosana. (G) Leaves with variegation patterns along veins (advein). (H–I) Leaf structures in a green area in (H) and a light area in (I). Note that the funnel-shaped chlorenchyma cells contact with adaxial epidermal cells tightly in green areas, but intercellular spaces (arrows) are found between these two cell layers in light areas in (A–I). (J–L) Oxalis corymbosa. (J) A leaf with varnish on the basal leaflets. (K–L) Leaf structures in a green area in (K) and a light area in (L). Note that the varnish leaf area in (L) with significantly larger adaxial epidermal cells and thicker outer cell walls (open arrow). Abbreviations: Chf, funnel-shaped chlorenchyma; Chs, spongy chlorenchyma; Eab, abaxial epidermis; Ead, adaxial epidermis; G, green area; L, light area. Arrows indicate intercellular spaces. Open arrow indicates the thicked cell wall. Scale bars = 50 μm.
Fig. 2. Variegated plants with light green (A, G) and white (D, J) patterns on adaxial leaf surfaces and comparative leaf structures between green and light areas. (A–C) Paphiopedilum concolor. (A) A leaf with light green patches covering the entire surface. (B–C) Leaf structures in a green area in (B) and a light area in (C). Note that the chlorenchyma tissue in the green area (B) has more chloroplasts. (D–F) Selaginella picta. (D) A shoot showing four rows of leaves along a branch; two median dorsal leaves and two outer ventral leaves. Note that each dorsal leaf has a white patch on the apex. (E–F) Leaf structures in a green area in (E) and a light area in (F), with free hand sections (insets). (G–I) Smilax bracteata subsp. verruculosa. (G) A leaf with sparsely scattered white patches on the adaxial surface. (H–I) Leaf structures in a green area in (H) and a light area in (I). (J–L) Valeriana hsuii. (J) A leaf with patches between veins. (K–L) Leaf structures in a green area in (K) and a light area in (L). Note that the funnel-shaped chlorenchyma cells contact with adaxial epidermal cells tightly in green areas, but intercellular spaces (arrows) are found between these two cell layers in light areas in (G–L). Abbreviations: Chf, funnel-shaped chlorenchyma; Chs, spongy chlorenchyma; DE, dorsal epidermal cell; Ead, adaxial epidermis; Elb, abaxial epidermis; G, green area; L, light area; VE, ventral epidermal cell. Scale bars = 50 μm.
microscope (TM3000, Hitachi, Tokyo, Japan) (Sheue et al., 2012). To measure cell size with Image J (National Institutes of Health, Bethesda, America), 30 cells were randomly selected from both green and light areas on one leaf from each of five individuals. A paired t-test (Zar, 2010) was used to test the difference between green and light areas.

For *P. concolor*, the chloroplast number in the funnel-shaped chlorenchyma of a light area seems less than that in the green area, as revealed by leaf semithin sections. For a quantitative comparison, one leaf segment from both the green and light areas of a leaf of each of four individuals was sampled. In each segment, cells showing full and complete funnel shape with clear chloroplasts were selected. In total, 11 funnel-shaped cells of each leaf segment were used to count chloroplast number per cell (totaling 44 cells for each green or light area). Data were then analyzed with a paired t-test (Zar, 2010).

The standard errors, SE, given in Table 2 were calculated at the leaf level. This means that the measurements on each leaf were used to calculate an average for the leaf. These averages were then used to calculate the standard deviation for a specific measurement (e.g. *Oxalis* adaxial epidermal cell size for normal leaves). The standard deviation was then divided by the square root of the number of leaves to give the standard error. This technique correctly accounts for variation between leaves in the traits measured (Zar, 2010).

## RESULTS

### The patterns of variegated leaves

Based on color variation of leaves of the studied taxa, four variegation colors are differentiated: silvery white (Fig. 1A), white (Figs. 1D, G; 2D, J), light green (Fig. 2A, G) and varnish, which is a shiny area on the top layer of mesophyll cells. The patterns of variegated leaves varies between leaves in the traits measured (Zar, 2010).

### white areas of *N. nipponica* strongly contrast with the dark green background (Fig. 1D), while the light areas of the leaf of *P. concolor* are light green (Fig. 2A). *Smilax bracteata* subsp. *verruculosa* also has similar light green patches, but with patches sparsely scattered on its leaves (Fig. 2G). The white areas on the leaf of *V. hsuii* appear between veins (Fig. 2J), like *S. bracteata* subsp. *verruculosa*.

Although belonging to the same genus, the two taxa of *Oxalis* have very different variegation features. The leaves of *O. acetosella* subsp. *griffithii* var. *formosana* have white areas on the adaxial surface mainly along the primary and secondary veins (Fig. 1G). In contrast, *O. corymbosa* has varnish at the base of each obcordate leaflet on its adaxial surface (Fig. 1J).

*Selaginella picta* is dorsiventral and anisophyllous, with striking white areas appearing on the apex of dorsal leaves (Fig. 2D). The variegation patterns of *S. picta* and *P. concolor* leaves can be observed from both adaxial and abaxial surfaces at the same locations, unlike the patterns on all other taxa studied, which shown variegation only on their adaxial leaf surfaces.

### Comparative anatomy

Six out of the eight taxa studied were found to have structural variegation. Only *P. concolor* and *S. picta* have pigment-related variegation (chloroplast type and chlorophyll type variegation, respectively). Of the six taxa with structural variegation, five (*B. aperta, N. nipponica, O. acetosella* subsp. *griffithii* var. *formosana, S. bracteata* subsp. *verruculosa* and *V. hsuii*) have the air space type structural mechanism. This mechanism is identified from the intercellular spaces found between the adaxial epidermal cells and the first layer of chlorenchyma cells in the light areas of the leaves of these species (Fig. 1C, F, I; Fig. 2I, L). In contrast, in the green areas of these leaves, the first layer of chlorenchyma cells is tightly connected to the adaxial epidermal cells (Fig. 1B, E, H; Fig. 2H, K).

In the leaf of *O. corymbosa*, two combined structural mechanisms were found to cause foliar variegation. In both green (Fig. 1K) and varnish (Fig. 1L) areas of *O. corymbosa* leaves, chlorenchyma cells contact the adaxial epidermal cells tightly. However, the thickness of the outer cell walls of adaxial epidermal cells in the green areas (3.09 ± 0.49 μm) (mean ± SE, here and below) is significantly thinner than in the varnish mark areas.
(6.23 ± 0.42 μm) (p = 0.003). Moreover, the cell size of adaxial epidermal cells in the green areas (72.11 ± 4.26 μm²) is significantly smaller than in the varnish areas (104.12 ± 7.83 μm²) (p = 0.029) (Table 2).

One species with pigment-related variegation, *P. conolor*, has chloroplast type variegation. In the variegated leaves of *P. conolor* (Fig. 2B, C), the chloroplast number in the top layer of mesophyll cells is significantly more than in the light area (7.64 ± 0.35) (p = 0.017). However, the chloroplast size in the top layer of mesophyll cells does not differ significantly (p > 0.05) between green (14.03 ± 0.77, maximum length) and light areas (13.86 ± 0.77) (p = 0.95).

The second species with pigment related variegation, *S. picta*, is the only species in this study to have the chlorophyll type mechanism, which is due to the absence of chloroplasts. In the dorsal leaves of *S. picta*, with a striking white segment near each leaf apex (Fig. 2F), chloroplasts were found only in the green areas (Fig. 2E).

**DISCUSSION**

Both pigment-related variegation and structural variegation were found in this study. The mechanisms of foliar variegation fall into four categories: air space type, epidermis type, chlorophyll type and chloroplast type. Among the eight taxa studied, air space type, a form of structural variegation, was found in five taxa (*B. aptera, N. nipponica, O. acetosella* subsp. *griffithii* var. *formosana*, *S. bracteata* subsp. *verruculosa* and *V. hsuii*). In Hara’s classical study of foliar variegation, air space type was found in 39 out of 55 taxa studied (Hara, 1957). More recently, air space type has been reported for *Aglaonema nitidum* (Fooshee and Henny, 1990), *Begonia rex* (Zhang et al., 2009) and several other *Begonia* species (Sheue et al., 2012). Collectively these studies suggest that the air space type probably is the most common mechanism of natural foliar variegation. It occurs widely in different families with distinct phylogenetic relationships in both monocots and eudicots.

Similar to most previous reports (Hara, 1957; Fooshee and Henny, 1990; Zhang et al., 2009; Sheue et al., 2012), this study found that the additional intercellular space causing foliar variegation occurs just beneath the epidermis. However, a recent study showed that intercellular space creating light areas on a leaf can also be located below adaxial water storage tissue (Sheue et al., 2012). Light areas are created by intercellular space due to reflections at the air-cell boundary, as explained by Snell’s law, above the chlorenchyma, and so a lower proportion of light is reflected from chlorophyll (Sheue et al., 2012).

In contrast, another structural variegation mechanism, the epidermis type, is rarely reported, as it is poorly understood. *Oxalis martiana* Zucc. (current name: *O. corymbosa* DC.) was the only species with epidermis type reported by Hara (Hara, 1957). The epidermis type was described by Hara as a “peculiarity of the epidermis.” Hara (1957, Fig. 2: III) depicts the leaves as having larger adaxial epidermal cells in “variegation parts” than in “normal parts” (1 to 0.34 in mean volume of epidermal cells) (Hara, 1957). Hara (1957) indicates that the variegation in this species occurs only on part of a leaf (Hara’s Fig. 3:14) specifically with “variegation parts” appearing at a leaf base. Because no further information is provided by Hara, we are not sure of the specific nature of the variegation pattern in *O. corymbosa* observed by Hara.

In our study, a shiny area (“varnish”) was found located at the base of each leaflet of *O. corymbosa*. Two distinctive structural differences were found between the varnish and normal green areas. They were (1) adaxial epidermal cells are larger in the varnish area than in the green area; and (2) the outer cell walls of adaxial epidermal cells are thicker in the varnish area than in the green area. We suggest these two differences both contribute to the epidermal type variegation mechanism. A previous study (Chen et al., 2017) showed that the epidermal type variegation can result from larger cells without a change in wall thickness. Combined with the present results from *O. corymbosa*, we recognized two subtypes of the epidermis type: the cell size subtype and the outer cell wall subtype. It is unclear exactly how the varnish appearance is created by these two mechanisms in *O. corymbosa*, but the thicker outer epidermal cell wall likely causes reflections from the leaf surface.

Pigment-related variegation was found in *P. conolor* and *S. picta*, which have chloroplast type and chlorophyll type mechanisms, respectively. The chloroplast number in the light areas of the leaves of *P. conolor* is less than that in the green areas. In the leaves of *S. picta*, no normal chloroplasts were found in the light areas. Such chlorophyll type variegation is very common in variegated ornamental plants, such as *Ficus pumila* L. ‘Sonny’ (Sheue et al., 2012) and *Zanthoxylum piperitum* (Hara, 1957). In contrast to the widely-recognized chlorophyll type, the chloroplast type is a relatively recent finding. It refers to the variegation caused by small but functional chloroplasts, few per cell (Chen et al., 2017).

Pigment related variegation potentially differs from structural variegation in allowing the variegation pattern to be evident from both the adaxial and abaxial sides of a leaf, as found here in the two taxa with chloroplast type and chlorophyll type mechanisms (*P. conolor* and *S. picta*). Sheue et al. (2012) proposed that an easy way to distinguish the structural mechanisms from the pigment-related variegation mechanisms is to observe the leaf from both sides with the naked eye under reflected light or with a stereoscope under transmitted light. Our observations of the eight taxa studied agree with this simple and easy method.
The trait of foliar variegation can be ‘persistent variegation’ (variegation maintained for the life of the plant) or ‘temporally present’ (variegation only at a particular life stage) (Chen et al., 2017). During our study, we found that *V. hsuii* usually displays variegated leaves before flowering. In this plant, the two pairs of leaves adjacent to its erect inflorescence are normal green leaves, while the leaves below them are variegated. A similar phenomenon has also been reported from *Sonerila heterostemon* (Melastomataceae) (Chen et al., 2017). Such transformation of green leaves before flowering in these variegated plants may encourage pollinator visits, but field studies to test this idea are needed.

According to our study and previous studies, structural variegation commonly occurs in nature in different major groups of vascular plants (Hara, 1957; Fooshee and Henny, 1990; Sheue et al., 2012; Chen et al., 2017). A recent study by Sheue et al. (2012) showed that structural variegation has almost no cost to photosynthesis, unlike chlorophyll type variegation. Such structural variegation leads to various foliar patterns that may provide visual signals discouraging herbivores and thus reducing herbivore damage (Campitelli et al., 2008). Another possible advantage of foliar variegation specifically associated with the structural mechanism is photoprotection from sunflecks (Esteban et al., 2008). However, evidence for these hypotheses is still scarce. This study contributes to a growing understanding of the contrivances by which plants are variegated, but variegation is under-reported and infrequently studied, yet variegation is found across the plant kingdom suggesting that circumstances have continually arisen that make this plant feature advantageous.

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LITERATURE CITED


