

Effect of Leaf Variegation Structure on Leaf Color in *Begonia gulinqingensis* Postprint

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Abstract

Using *Begonia gulinqingensis* as experimental material, this study investigated the causes of leaf spot formation through analysis of leaf morphological characteristics, upper epidermal optical properties, tissue structure, chlorophyll content, and chlorophyll fluorescence parameter F_v/F_m . The results showed that the frequency and number of leaf spots in *Begonia gulinqingensis* exhibited no obvious pattern, but their location was relatively stable, with spots occurring mainly between the two main veins opposite the petiole. Spotted regions displayed two light reflection patterns: punctate reflection and polygonal reflection; palisade tissue cells were nearly isodiametric and circular, loosely arranged, with air spaces present between them and the upper epidermal cells. Non-spotted regions exhibited only punctate reflection, with palisade tissue cells being funnel-shaped, tightly arranged, and lacking air spaces between them and the upper epidermal cells. Chloroplasts in both spotted and non-spotted regions possessed densely stacked grana and abundant thylakoid membranes; chlorophyll a, chlorophyll b, and total chlorophyll contents in spotted regions were only 24.9%, 25.2%, and 25.1% lower than those in non-spotted regions, respectively. The chlorophyll fluorescence parameter F_v/F_m value was 0.793 in spotted regions and 0.790 in non-spotted regions. Although chlorophyll content in spotted regions was slightly lower than that in non-spotted regions, chloroplast structure remained intact, and chlorophyll fluorescence parameters showed no significant difference from non-spotted regions. The air spaces between the upper epidermis and palisade tissue cells in spotted regions could cause secondary reflection of light when reaching the green tissues, forming white polygonal light reflections at the edges of leaf epidermal cells that made these areas appear whiter than surrounding normal leaf regions. Based on these results, it can be inferred that the formation of light green patches in *Begonia gulinqingensis* is related to specialized leaf structure.

Full Text

Preamble

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Title: Effect of Foliar Variegation Structure on Leaf Color in *Begonia gulinqingensis*

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Abstract

Using *Begonia gulinqingensis* as experimental material, variegated leaves were analyzed for leaf morphological features, optical properties of the adaxial surface, tissue structure, chlorophyll content, and chlorophyll fluorescence parameter Fv/Fm to investigate the causes of pale green patch formation. The results showed that: (1) The frequency and number of patches on a leaf showed no obvious regularity, but their location was relatively stable, occurring primarily between the two main veins opposite the petiole. (2) Two light reflection patterns were observed in variegated areas: spotted pattern (SP) and polygonal pattern (PP). Palisade tissue cells were nearly equiaxed and circular, arranged loosely with intercellular spaces between the adaxial epidermal cells and palisade tissue cells. Non-variegated areas exhibited only spotted pattern reflection, with palisade tissue cells being funnel-shaped, arranged tightly, and lacking intercellular spaces with the epidermal cells. (3) Chloroplasts from both variegated and non-variegated areas showed dense stacking of grana and abundant thylakoid membranes. Chlorophyll a, b, and total chlorophyll contents in variegated areas were only 24.9%, 25.2%, and 25.1% lower than those in non-variegated areas, respectively. (4) The chlorophyll fluorescence parameter Fv/Fm was 0.793 in variegated areas and 0.790 in non-variegated areas. Although chlorophyll content in variegated areas was slightly lower, chloroplast structure remained intact and chlorophyll fluorescence parameters showed no significant difference from non-variegated areas. Based on these results, we infer that the intercellular space between the adaxial epidermis and palisade tissue cells may cause secondary reflection when light reaches the green tissue, creating white polygonal light reflection at the edges of epidermal cells rather than the white spot reflection typical of normal leaves. This makes the area appear whiter than surrounding normal leaf tissue, suggesting that the pale green patches in *B. gulinqingensis* are related to this special leaf structure.

Keywords: *Begonia gulinqingensis*, adaxial surface optical property, leaf structure, chlorophyll content, Fv/Fm

Introduction

Begonia L. is an important horticultural plant widely cultivated worldwide, valued for both its flowers and foliage. Its rich leaf coloration is rare among plants, with approximately 15,000 horticultural varieties, most of which are variegated types. China is rich in wild *Begonia* resources, with variegated species accounting for about 41.4% of the total. The size, shape, position, and color of leaf variegation are diverse, with some species named after their variegation patterns, such as *B. cathayana* Hemsl. and *B. versicolor* Irmsch. (Cui & Guan, 2013).

Plant leaf variegation refers to the appearance of differently colored spots or stripes on leaf surfaces (Li, 2010). Hara (1957) studied 55 variegated species across 24 families and classified the causes of variegation into two categories with four types: pigment types (including chlorophyll and pigment types) and structural types (including epidermal and air-space types). Currently, most research focuses on pigment-type variegation (Aluru et al., 2001; Jiang et al., 2004), while structural variegation has been less studied (Fooshee & Henny, 1990; Tsukaya et al., 2004). Leaves are the most important sites for photosynthesis, and changes in pigment content and tissue structure can cause leaf color changes while also affecting photosynthetic physiological indicators (Wang et al., 2016).

Chlorophyll-type variegation mainly results from chloroplast structural mutations (degradation of grana lamellae, reduced stroma lamellae, chloroplasts lacking membrane structures or thylakoids, formation of osmiophilic granules, incomplete chloroplast structures, etc.), which impede chlorophyll synthesis and cause white or yellow leaf coloration. The tissue structure of such variegated areas shows no significant difference from normal green areas, but chlorophyll content is significantly lower, and chlorophyll fluorescence parameter F_v/F_m is reduced (Fan et al., 2006; Chang et al., 2008; Wang, 2012; Wei, 2013; Yang, 2015; Li et al., 2016). Pigment-type variegation causes red, purple, or other colored leaves due to anthocyanin presence. The leaf structure of such variegated leaves shows no obvious difference from normal green leaves, with lower chlorophyll content but higher anthocyanin content (Yuan et al., 2010; Du et al., 2017). Epidermal cell variations cause light interference, diffraction, and refraction, while air-space structures cause secondary light reflection. Both alter the light path on the leaf surface and within the leaf, affecting absorption and reflection spectra and ultimately causing leaves to appear blue-tinged, white, silver-white, light green, or silver-green. These structural variegations constitute physical colors (Zhang et al., 2009; Wang et al., 2012; Sheue et al., 2012). Structural variegation shows different tissue structures between variegated and normal green areas—some plants have thicker epidermal cells (Hara, 1957), while others have thin-film interference filters, multi-layer film structures, or iridoplasts in the epidermis (Wang et al., 2012). In *Schismatoglottis calyptrata*, cyclamen, and *Arum italicum*, air spaces exist between palisade tissue cells and the upper epidermis. Chlorophyll content in structural variegation is generally slightly lower in variegated areas than in non-variegated areas. Chlorophyll fluorescence parameter F_v/F_m may be slightly higher or lower in variegated areas compared to

non-variegated areas, but without significant differences (Tsukaya et al., 2004; Konoplyova et al., 2008; Rocca et al., 2011; Sheue et al., 2012). Recent studies have shown that leaf color can be influenced by multiple mechanisms working together, such as pigment content and air-space structure jointly affecting leaf color in *Actinidia kolomikta* (Wang et al., 2016), and epidermal cells, intercellular spaces, mesophyll cells, chloroplast variations, and crystals collectively enhancing white spots in *Blastus cochinchinensis* seedlings (Chen et al., 2017).

Colorful variegation not only improves ornamental traits but also enhances plant ornamental and economic value. *Begonia gulinqingensis* S. H. Huang & Y. M. Shui is an evergreen herb with circular leaves, dark green adaxial surfaces, and slightly reddish abaxial surfaces. Individual plants may have leaves with irregular light green patches or no patches. Variegation is present when young leaves emerge. This species is endemic to Yunnan and currently endangered. Its unique variegation pattern is rare among foliage begonias and has extremely high ornamental and development value (Ma et al., 2005). Currently, few studies have reported on the leaf variegation of *B. gulinqingensis*. This study investigates the tissue structure characteristics, chlorophyll content, and chlorophyll fluorescence parameter F_v/F_m of variegated leaves in *B. gulinqingensis* to explore the causes of variegation formation and provide a theoretical basis for elucidating the mechanism of variegation development.

Materials and Methods

1.1 Plant Material

Begonia gulinqingensis (*B. gulinqingensis*) was cultivated at the Bulb and Perennial Flower Breeding Base of the Flower Research Institute, Yunnan Academy of Agricultural Sciences. This study used variegated and non-variegated tissues from variegated leaves as experimental material.

1.2 Experimental Methods

1.2.1 Observation of Leaf Variegation Morphology Beginning in April 2016, we observed the frequency, number, and location of leaf variegation in *B. gulinqingensis*. Both variegated and non-variegated leaves were collected for cutting propagation, and the occurrence of variegation in the cutting-derived plants was statistically analyzed.

1.2.2 Observation of Leaf Epidermal Optical Properties After cleaning, leaves were placed on filter paper and observed under a dissecting microscope equipped with a mercury lamp in a dark room (Zhang et al., 2009). Epidermal brightness and cell reflection patterns were used to evaluate leaf optical properties.

1.2.3 Tissue Structure Observation For semi-thin section preparation, we followed the method of Gong et al. (2014). Mature variegated leaves were sam-

pled from both variegated and non-variegated areas. Samples measuring $1 \times 1 \times 1$ mm were excised with a blade (three replicates). The samples were fixed in 2.5% glutaraldehyde, washed three times with $0.1 \text{ mol} \cdot \text{L}^{-1}$ PBS buffer for 15 minutes each, post-fixed in 1% osmium tetroxide for 1.5-2 hours, and rinsed again three times with $0.1 \text{ mol} \cdot \text{L}^{-1}$ PBS buffer for 15 minutes each. Dehydration was performed using an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, 100%) for 15 minutes each, followed by a second dehydration in 100% ethanol for 20 minutes and acetone for 20 minutes. Infiltration was conducted with embedding agent:acetone (1:1) for 1 hour at room temperature, then embedding agent:acetone (3:1) for 3 hours at room temperature, and finally pure embedding agent overnight. The embedding agent was transferred to 0.5 mL centrifuge tubes or embedding molds, samples were placed inside, and polymerization was performed at 70°C for 12 hours or 60°C for 24 hours (two cycles). Sections of 1 μm thickness were cut, stained with toluidine blue, and observed under a microscope.

For ultrathin sections, the same method as for semi-thin sections was used, but with section thickness of 50-70 nm. Sections were stained first with uranyl acetate for 5-10 minutes, rinsed, then stained with lead citrate for 5 minutes, rinsed again, dried under a heat lamp, and observed and photographed using transmission electron microscopy.

1.2.5 Chlorophyll Content Determination Chlorophyll content was determined following the method of Li et al. (2000). Leaf material (0.2 g, with veins removed) was weighed and cut into small pieces. Since the variegated area mass from a single leaf was insufficient for 0.2 g, we pooled samples from different plants and leaves. Twelve variegated leaves were collected from ten plants and grouped into three sets of four leaves each. Both variegated and non-variegated tissues (0.2 g each) were ground in a mortar with a small amount of quartz sand, calcium carbonate powder, and 2-3 mL of 95% ethanol until a homogenate formed. The homogenate was filtered, rinsed, and brought to a final volume of 25 mL with ethanol solution. A spectrophotometer was used to measure absorbance of the chlorophyll solution at 665, 649, and 470 nm, and chlorophyll concentration was calculated. Each sample was replicated six times, and mean values were obtained.

1.2.6 Determination of Chlorophyll Fluorescence Parameter F_v/F_m

A chlorophyll fluorescence imaging system was used to measure fluorescence parameters in variegated and non-variegated areas of *B. gulinqingensis* leaves. Mature variegated leaves were dark-adapted by wrapping in aluminum foil for 20 minutes, after which fluorescence parameters F_v/F_m were measured, including minimum fluorescence (F_o) and maximum fluorescence (F_m) in the dark-adapted state, with $F_v = F_m - F_o$. Nine replicates were performed.

Results

2.1 Leaf Variegation Morphology

Initial observations revealed that the frequency and number of leaf patches in *B. gulingqingensis* showed no obvious pattern among different plants, but their location was relatively stable. Individual plants simultaneously produced both variegated and non-variegated leaves. Cutting propagation using both leaf types resulted in offspring that produced both variegated and non-variegated leaves. The number of patches on variegated leaves was variable, typically one but sometimes two or more. When only one patch was present, it usually occurred between the two main veins opposite the petiole [Figure 1: see original paper]-A. When two or more patches were present, one always occurred between the main veins opposite the petiole, with additional patches distributed among surrounding main veins; patches rarely crossed main veins [Figure 1: see original paper]-B, C. Patches were present when young leaves emerged and increased in area as leaves expanded, stabilizing in size and shape when leaves reached maturity. The non-variegated areas of variegated leaves showed the same color as non-variegated leaves, with dark green adaxial surfaces [Figure 1: see original paper]-D and red veins on the abaxial surface [Figure 1: see original paper]-E.

2.2 Leaf Surface Optical Properties

Stereomicroscope observation [Figure 2: see original paper]-A, B revealed two light reflection patterns in variegated leaves of *B. gulingqingensis*: spotted pattern (SP) and polygonal pattern (PP). In the light green variegated areas, adaxial epidermal cells exhibited not only spotted reflection (SP) but also faint bright rings around cell edges that reflected cell shape. Since most cells were irregular polygons, this reflection pattern was termed polygonal reflection (PP) [Figure 2: see original paper]-C, D. Non-variegated areas showed only spotted pattern reflection, with a bright spot at the center of each adaxial epidermal cell [Figure 2: see original paper]-C, D.

2.3 Tissue Structure and Ultrastructure

Semi-thin sections showed that in light green variegated areas, adaxial epidermal cells were relatively flat with occasional small protrusions, while non-variegated area cells had rectangular protrusions and were slightly larger. Palisade tissue cells in variegated areas were smaller, irregularly circular, loosely arranged, and contained few or very few chloroplasts. A large intercellular space formed an “air chamber” between the palisade tissue layer and epidermal cells. Spongy tissue cells were similar in size and shape to palisade tissue cells, arranged in two loose layers, and contained chloroplasts [Figure 3: see original paper]-A. In non-variegated areas, palisade tissue cells were larger, cylindrical, tightly arranged, and contained numerous chloroplasts, with no space between them and epidermal cells. Spongy tissue consisted of two tightly arranged layers of irregularly circular cells; the layer adjacent to palisade tissue was smaller and contained

chloroplasts, while the lower layer was larger and lacked chloroplasts [Figure 3: see original paper]-B. Both variegated and non-variegated areas consisted of four cell layers. Variegated area cells were uneven in size and irregularly arranged, while non-variegated area cells were uniformly sized, neatly layered, and tightly arranged. Non-variegated area cells were slightly larger, resulting in similar overall thickness between variegated and non-variegated areas.

Ultrathin sections revealed that chloroplasts in variegated areas were elliptical and contained starch grains [Figure 4: see original paper]-A, B, while those in non-variegated areas were slender and elliptical with clear grana lamellae [Figure 4: see original paper]-C, D. Although chloroplast ultrastructure differed between the two areas, both had intact structures with abundant thylakoid membranes and densely stacked grana.

2.4 Chlorophyll Content and Chlorophyll Fluorescence Parameter Fv/Fm

Chlorophyll content measurements showed that chlorophyll a, b, and total chlorophyll contents in variegated areas were lower than in non-variegated areas, [Figure 5: see original paper]-A, B, C. Variegated area chlorophyll a, b, and total chlorophyll contents were 24.9%, 25.2%, and 25.1% lower than non-variegated areas, respectively. Semi-thin section observations revealed that palisade tissue cells in variegated areas contained significantly fewer chloroplasts than non-variegated areas, but spongy tissue cells contained more chloroplasts, compensating for the reduced chloroplast content in green tissue cells.

Measurements of chlorophyll fluorescence parameter Fv/Fm in isolated variegated and non-variegated areas showed values of 0.793 and 0.790, respectively, with no significant difference [Figure 5: see original paper]-D. Fv/Fm represents the maximum photochemical efficiency of PSII, reflecting quantum yield when all PSII reaction centers are open, and can be used directly as an indicator of primary photochemical efficiency. Chloroplast ultrastructure in variegated areas was intact. Although total chlorophyll content was 25.1% lower than in non-variegated areas, functional chloroplasts in variegated areas could adequately absorb light energy, resulting in similar maximum photochemical efficiency to non-variegated areas. Therefore, *B. gulingqingensis* can maintain normal photosynthesis despite having variegation. Since this species grows in the understory of tropical rainforests, this leaf color pattern represents a long-term evolutionary adaptation to low-light environments.

Discussion and Conclusion

Previous studies have identified eight *Begonia* species with structural variegation caused by air-space structures: *B. rex* Putz., *B. formosana* (Hayata) Masamune, *B. hemsleyana* Hook. f., *B. versicolor* Irmsch., *B. chlorosticta* Sands, *B. diadema* Linden, *B. pustulata* Liebm, and the cultivar *B. 'K030960'*. In seven of these species, air-space structures exist between palisade tissue cells and the upper

epidermis, while in cultivar *B.* 'K030960', air spaces occur between water-storage tissue cells beneath the upper epidermis and palisade tissue cells (Zhang et al., 2009; Sheue et al., 2012). In *B. gulinqingensis*, we observed air-space structures between adaxial epidermal cells and palisade tissue cells, with epidermal cells in variegated areas being slightly smaller than those in non-variegated areas. Zhang et al. (2009) and Sheue et al. (2012) found no differences in epidermal cell size between variegated and non-variegated areas in *B. rex*, *B. hemsleyana*, *B. versicolor*, *B. chlorosticta*, *B. pustulata*, and *B.* 'K030960', while Sheue et al. (2012) observed slightly smaller epidermal cells in variegated areas of *B. formosana* and *B. diadema*. This indicates that epidermal cell size differences between variegated and non-variegated areas are not an absolute characteristic of *Begonia* species.

Palisade tissue cells in variegated areas of *B. gulinqingensis* were circular with fewer chloroplasts, while those in non-variegated areas were funnel-shaped with numerous chloroplasts, similar to the other eight air-space variegated *Begonia* species described by Zhang et al. (2009) and Sheue et al. (2012). However, in those eight species, spongy tissue cells in non-variegated areas were loosely arranged and similar in size and morphology to those in variegated areas, whereas in *B. gulinqingensis*, non-variegated area spongy tissue cells were tightly arranged with distinct differences in morphology and size compared to variegated areas. Ultrastructural observations showed that although chloroplast shapes differed between variegated and non-variegated areas in *B. gulinqingensis*, both had intact structures with abundant thylakoid membranes and densely stacked grana, and starch grains were observed in variegated areas, consistent with previous studies (Zhang et al., 2009; Sheue et al., 2012; Chen et al., 2017).

Regarding chlorophyll content, variegated areas of *B. gulinqingensis* had slightly lower chlorophyll content than non-variegated areas, but not enough to affect leaf color, as variegated areas remained green, only lighter in shade. A similar phenomenon was observed in white leaves of *Actinidia kolomikta* (Wang et al., 2016). In this study, chlorophyll fluorescence parameter Fv/Fm in variegated areas of *B. gulinqingensis* showed little difference from non-variegated areas. Three other *Begonia* species—*B. formosana*, *B. diadema*, and *B. pustulata*—have also been reported to show no significant difference in Fv/Fm values between variegated and non-variegated areas (Sheue et al., 2012). Rocca et al. (2011) found no significant differences in maximum photochemical efficiency (Fv/Fm) of PSII among differently colored leaves of *Arum italicum*. Chen et al. (2017) reported no significant difference in Fv/Fm between variegated and non-variegated areas in *Blastus cochinchinensis*, though Fv/Fm values in non-variegated areas of variegated leaves were higher than those in non-variegated leaves.

In summary, leaf variegation in *B. gulinqingensis* belongs to the air-space structural type. The presence of air-space structures causes secondary reflection when light reaches green tissue, creating white polygonal light reflection at the edges of epidermal cells rather than the white spot reflection typical of normal

leaves, making the area appear whiter than surrounding normal tissue and thus forming variegation (Hara, 1957; Zhang et al., 2009). This is consistent with our observations of leaf epidermal optical characteristics and similar findings in *B. rex* (Zhang et al., 2009). Additionally, Sheue et al. (2012) found that *B. chlorosticta* with air-space variegation had green leaf margins, red abaxial surfaces, dark green adaxial surfaces, and light green patches. This was attributed to the combination of red and green on the abaxial surface creating dark green on the adaxial surface, which appears light green rather than white when air-space structures are present. Young leaves of *B. gulinqingensis* are red when they first emerge, gradually turning green while maintaining red veins on the abaxial surface. Since its variegation is also of the air-space type, we speculate that the color development mechanism of its light green patches is similar to that of *B. chlorosticta*.

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