

***Actinidia kolomikta* leaf colour and optical characteristics**

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Abstract

Actinidia kolomikta (Rupr. & Maxim.) Maxim. leaves showed dramatic colour changes during plant growth phases, and we studied structure and optical properties of variegated leaves. Leaf surface cells were smooth, and there were no surface appendages (wax or trichomes) observed in variegated leaves. Palisade tissue cells in white and pink leaves were looser and contained relatively fewer chloroplasts. White leaves contained many intercellular spaces between the epidermal and mesophyll cells or within the palisade cell layer. Variegated leaves had three distinct radiation reflection patterns: a bright white area, a spotted pattern, and a polygonal pattern. Reflectance at 450 - 1100 nm from the adaxial surface of white leaves was greater than that of green leaves, but anthocyanin accumulation in pink leaves decreased the reflectance at 500 - 600 nm. When variegated leaves turned green, the reflectance at 500 - 600 nm increased. On abaxial surfaces, the reflectance of variegated leaves was similar to green leaves at 450 - 700 nm. In conclusion, reflection patterns and the formation of variegated leaves of *A. kolomikta* were significantly correlated with the leaf anatomy. The white and pink colours of leaves were a result of an internal reflection between air spaces and cells in the leaves, chlorophyll deficiency in palisade tissue, and anthocyanin accumulation. Variegated leaves turned green when the chlorophyll content in palisade tissue increased.

Additional key words: anthocyanin, chlorophyll, leaf anatomy, reflectance, spectrum characteristics, variegated leaves.

Introduction

Variegated plants are popular ornamental plants. Plant variegations are usually gene mutations or caused by environmental factors such as high irradiation and low temperature (Esteban *et al.* 2008, Sakamoto *et al.* 2009). Variegation is defined as the presence of red, purple, yellow, pale-green, or silver-white parts. These coloured areas may have regular or irregular patterns.

Leaves of *Actinidia kolomikta* are initially green. They develop white patches during late-spring or early summer, and by mid- to late-summer these white leaves turn pink or light green. *A. kolomikta* displays an amazing variety of leaf colour during the development, which is rare among plant species. Previous research indicated that the silver and white leaves in some plant species are caused by the presence of trichomes and waxes (Reicosky

and Hanover 1978, Karabourniotis *et al.* 1999, Liakopoulos *et al.* 2006), which reflect radiation. Other studies reported that epidermal accumulation of pigments (such as anthocyanin) (Esteban *et al.* 2008, Zeliou *et al.* 2009), chlorophyll deficiency (Yu *et al.* 2007, Sakamoto *et al.* 2009), and the presence of extensive intercellular air spaces (Rocca *et al.* 2011) can cause a leaf variegation. These studies do not provide information on surface optical properties that occur during leaf colouring. The theoretical basis of variegation has not yet been established and a relationship between optical properties and leaf structure remains unclear. We characterized the leaf colouring, structure, and surface optical properties of variegated leaves of *A. kolomikta*. These data may suggest some possible mechanisms leading to foliar variegation.

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Abbreviations: A - absorbance; ARI - anthocyanin reflectance index; ChlNDI - chlorophyll normalized difference vegetation index; CRI - carotenoid reflectance index; PP - polygonal pattern; R - reflectance; SEM - scanning electron microscopy; SP - spotted pattern; T - transmittance.

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Materials and methods

Actinidia kolomikta (Rupr. & Maxim.) Maxim. is a deciduous woody creeping vine. The plant is a long-lived and can grow up to 8 - 10 m. *A. kolomikta* is the hardiest species in the genus surviving winter temperatures of -40 °C. Some of its leaves undergo seemingly random variegation (green leaves develop patches of pink and white) during late spring to mid-summer. A study area was the National Field Gene Bank for *A. kolomikta* of the Institute of Special Wild Economic Animal and Plant Science, Jilin (N 44° 04' and E 126° 05'), China. Annual precipitation was 679 mm and the annual temperature range from +35 to -40 °C in the region. Plants in the experimental area received direct sunlight up to 1 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Average day/night summer temperature was 30/10 °C and relative humidity 60/85 %. Thirty (10 pink, 10 white, and 10 green), south-facing leaves were used for the study. Measurements began on May 15, 2014. Similar dimensions and a similar exposure to sunlight were the criteria for leaf selection.

Leaves were washed with distilled water, placed on glass slides, observed, and photographed with a digital microscope (*ECLIPSE 80i*, *Nikon*, Tokyo, Japan) equipped with a halogen lamp (12 V, 100 W, and 3 100 K) in a dark room (Zhang *et al.* 2009). The leaf surface was placed vertically to the light source of the microscope. Green, white, pink, and light green leaves were selected for these observations.

Reflectance (R) was measured with 10 leaves at different development stages using a bifurcated fiber optic cable and a leaf-clip of an *Unispec* spectrometer (*PP Systems*, Amesbury, MA, USA). The leaf was irradiated from one side with a tungsten halogen lamp in

the spectrometer using the bifurcated fiber cable. Transmittance (T) was measured according to a method of Konoplyova *et al.* (2008). Absorbance (A) was calculated as $A = 1 - (R + T)$. The following reflectance indices were derived from spectral reflectance curves: the chlorophyll normalized difference vegetation index, (ChlNDI) which can estimate a relative chlorophyll content [ChlNDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$; Gitelson and Merzlyak 1994, Blackburn 1998], the carotenoid reflectance index (CRI) which is a measure of carotenoid content [CRI = $(1/R_{510}) - (1/R_{550})$; Gitelson *et al.* 2002], and the anthocyanin reflectance index (ARI) which is a measure of anthocyanin content [ARI = $R_{800} \times \{(1/R_{550}) - (1/R_{700})\}$; Gitelson *et al.* 2001].

Sample preparation to produce semi-thin sections and scanning electron microscopy (SEM) was performed as described by Tsukaya (2004). Semi-thin sections were stained with 1 % (m/v) toluidine blue and observed under an optical microscope (*ECLIPSE 80i*). Photomicrographs were taken using a *Zeiss Axiolab* with a digital camera (*DXM1200*, *Nikon*). Leaf cells were observed using an SEM (*JSM-6610*, *JEOL*, Tokyo, Japan) at 30 kV at a magnification of 500 \times .

Statistical analysis employed a *SAS* (*JMP 6.0, SAS Institute*, Cary, NC, USA) software. For data sets having parametric distributions, the significance of differences between treatment means was determined using the Student's *t*-test ($P < 0.05$). The leaf reflectance was the mean of 10 independent measurements. The leaf transmittance was the mean of three independent measurements.

Results

Adaxial epidermal cells were smaller in white and red leaves than in green leaves (Fig. 1). Leaf epidermal cells were smooth, and waxes or trichomes were not present. Palisade parenchyma of normal green leaves consisted of an established layer of packed and elongated cylindrical cells (Fig. 2A) that were in a close contact with the adaxial epidermis. In variegated leaves, palisade parenchyma cells were rounded or outlined with irregular rings (Fig. 2B). In contrast to normal green leaves (Fig. 2A), the palisade parenchyma cells in variegated leaves were loose, and contained fewer chloroplasts. Spongy mesophyll was well-developed in variegated leaves (Fig. 2B). Cells containing chloroplasts in variegated leaves were chiefly present in spongy mesophyll layers (Fig. 2B). Numerous intercellular spaces in variegated leaves were observed between the epidermal and mesophyll cells or within the palisade parenchyma (Fig. 2B).

Variegated leaves showed three distinct patterns: one

bright white area with no distinct pattern, a spotted pattern (SP) formed in the centre of epidermal cells, and a polygonal pattern (PP) composed of white polygons formed around the edges of epidermal cells (Fig. 3). Within white areas, we didn't observe SP and PP. The SP was observed in both pink, light green, and normal green areas. The PP was much stronger in pink and light green than in normal green areas.

We analyzed leaf optical properties by documenting a reflectance spectrum over the range of 450 - 1100 nm of juvenile green, green, juvenile white, white, light pink, pink, and turning green leaves (Fig. 4). The adaxial reflectance of young white leaves was similar to that of fully expanded white leaves. However, the reflectance of young white leaves was higher at 500 - 700 nm than of the other colour leaves on abaxial surfaces.

On adaxial surfaces, the reflectance of variegated leaves was higher than that of green leaves at 450 - 1100 nm

(Fig. 4). Over the entire sampling period, the reflectance of white leaves was mostly highest. The reflectance of variegated leaves changed significantly during the period of leaf colouring. The reflectance of variegated leaves at 500 - 600 nm decreased significantly during the early

pink and pink periods compared to the white period. With variegated leaves turning light green, the reflectance of variegated leaves at 500 - 600 nm increased significantly compared to the pink period. On abaxial surfaces, the reflectance was less than on adaxial

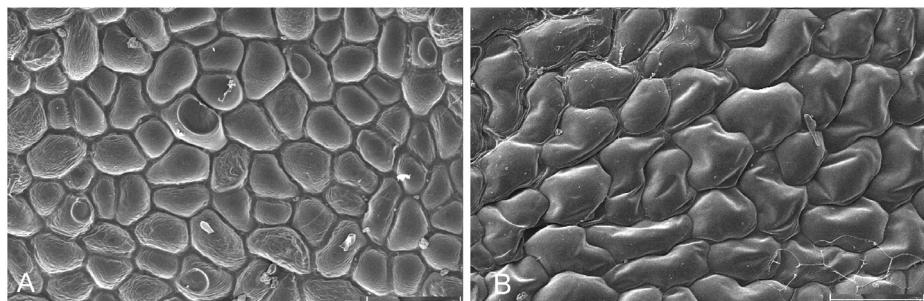


Fig. 1. Scanning electrom microscopy micrographs of adaxial epidermis of variegated leaves of *A. kolomikta*. A - white area, B - green area. Adaxial epidermal cells were smaller in white and red leaves than in green leaves. Bars = 50 μ m.

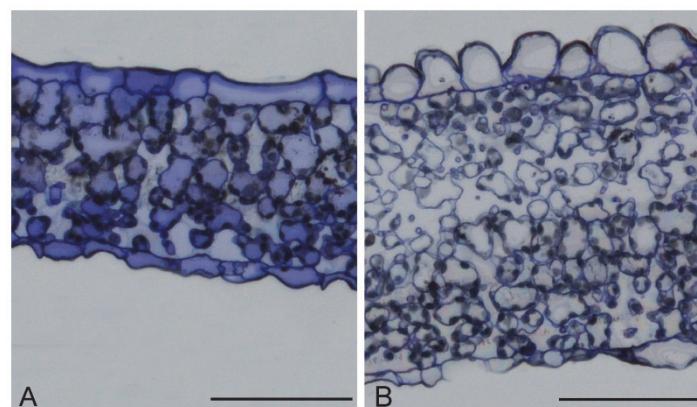


Fig. 2. Transverse sections from green leaves (A) and white leaves (B) of variegated *A. kolomikta*. Scale bars = 50 μ m.

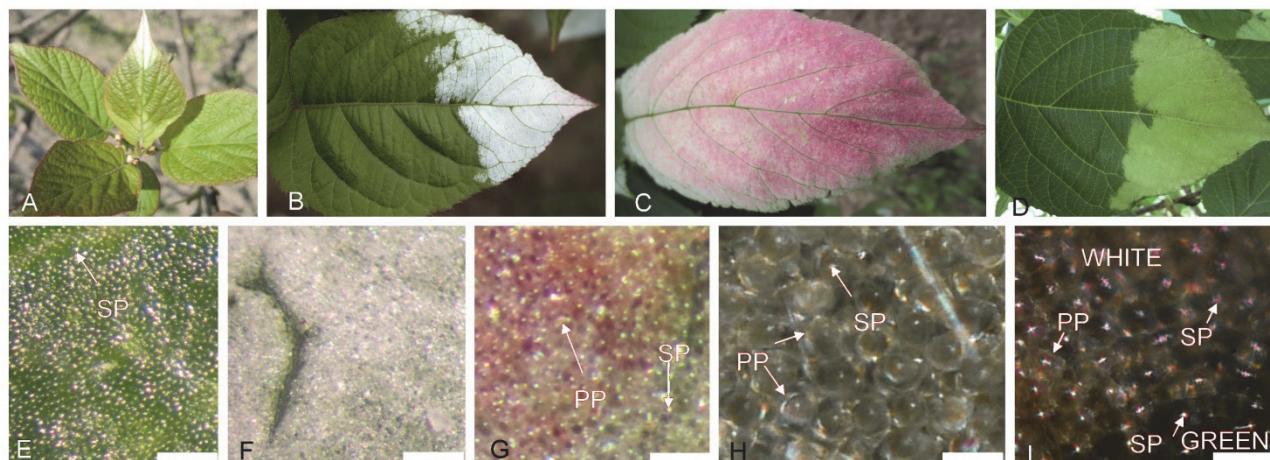


Fig. 3. Colour changes during leaf development of variegated leaves: the juvenile period (A), white period (B), pink period (C), and turning green period (D). Adaxial surface patterns of variegated leaves: the green surface (E), white surface (F), pink surface (G), and turning green surfaces (H and I). Adaxial epidermal cells in the pink and light green areas show irregular white rings (a polygonal pattern) under reflected light outlining the cells. However, in the white area, adaxial epidermal cells show dazzling lustre and no pattern. Scale bars: E, F = 1 000 μ m; G, H, I = 50 μ m. SP - spotted pattern, PP - polygonal pattern.

surfaces. The reflectance between variegated and green leaves was similar at 450 - 700 nm but significantly increased at 700 - 1100 nm (Fig. 4).

The shape of the transmittance curves was similar between white and green leaves at 480 and 680 nm (Fig. 5). Pink leaves had the lowest transmittance but the transmittance increased during the green transformation period.

Comparison of the adaxial surface absorbances of

variegated and green leaves demonstrated that the absorbance increased gradually with the variegated leaf age. In variegated leaves turning light green, the reflectance of variegated leaves at 500-600 nm decreased significantly compared to the pink period. The absorbance of normal green leaves was highest.

As concerns parameters calculated from the reflectance curves on adaxial surfaces, the ChlNDI increased gradually with the green leaf age. This

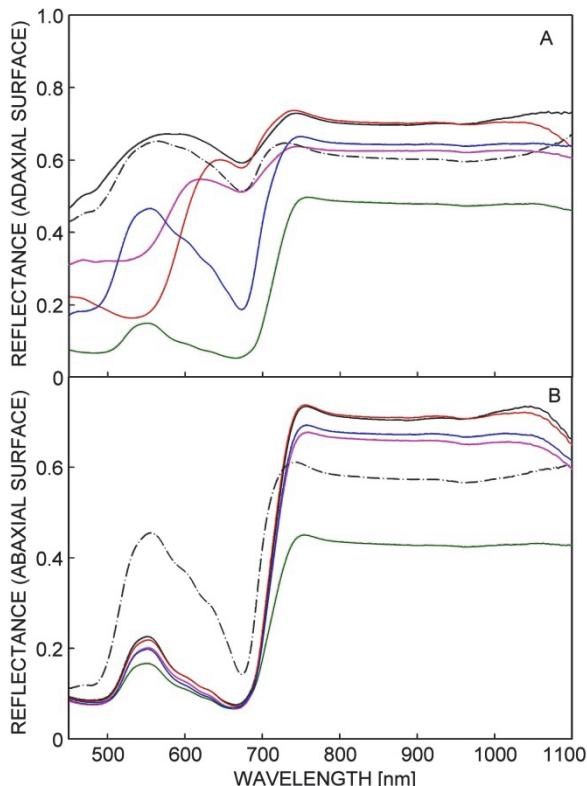


Fig. 4. Reflectance spectra of adaxial (A) and abaxial (B) surfaces of different *A. kolomikta* leaves: green leaves (green lines), juvenile white leaves (black broken lines), white leaves (black lines), pink leaves (red lines), light pink leaves (pink lines), and turning green leaves (blue lines).

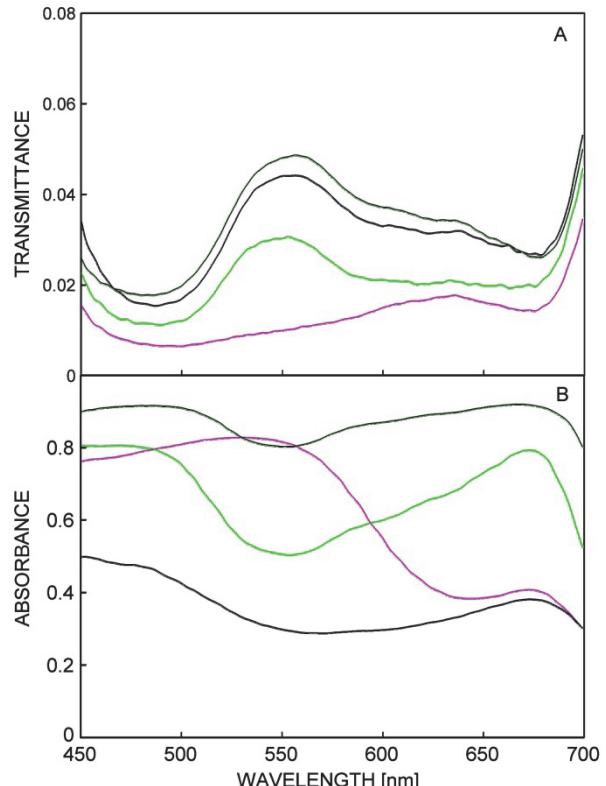


Fig. 5. A - Transmittance spectra of the adaxial surface of different *A. kolomikta* leaves: green leaves (green line), white leaves (black line), turning green leaves (light green line), pink leaves (pink line). B - The absorbance of photosynthetically active radiation (450 - 700 nm) in the same leaves as in A.

Table 1. Surface reflectance characteristics of different leaves of *Actinidia kolomikta*. Means \pm SD, $n = 10$ independent experiments. ANOVA was performed for pairs of corresponding values between variegated and green leaves.

Surface	Parameters	Juvenile green	Green	Juvenile white	White	Light pink	Pink	Turning green
Adaxial	ARI	0.47 \pm 0.10 a	0.19 \pm 0.09 b	-	-	0.78 \pm 0.33 c	3.16 \pm 0.74 d	-
	ChlNDI	0.25 \pm 0.02 b	0.51 \pm 0.01 a	0.03 \pm 0.01 e	0.05 \pm 0.01 d	0.05 \pm 0.01 ed	0.04 \pm 0.01 ed	0.18 \pm 0.03 c
	CRI	5.19 \pm 0.39 a	5.42 \pm 0.44 a	0.28 \pm 0.06 c	0.19 \pm 0.02 dc	0.22 \pm 0.04 dc	-	1.46 \pm 0.31 b
Abaxial	ARI	0.66 \pm 0.21 a	0.23 \pm 0.07 bc	-	0.12 \pm 0.26 c	0.24 \pm 0.04 bc	0.30 \pm 0.07 d	0.23 \pm 0.12 bc
	ChlNDI	0.22 \pm 0.02 d	0.36 \pm 0.02 c	0.21 \pm 0.02 d	0.43 \pm 0.02 a	0.42 \pm 0.03 ab	0.40 \pm 0.02 b	0.42 \pm 0.05 ab
	CRI	3.59 \pm 0.63 b	4.44 \pm 0.39 a	3.55 \pm 0.12 d	4.70 \pm 0.01 a	4.89 \pm 0.36 a	4.46 \pm 0.33 a	4.83 \pm 0.28 a

parameter was very similar in variegated leaves but not in turning green leaves. When variegated leaves turned green, the ChlNDI and CRI significantly increased. With variegated leaf development, the ARI increased gradually

Discussion

The colour of leaves is not determined only by a pigment type, content, and distribution (Neill and Gould 1999, Gould *et al.* 2000, Konoplyova *et al.* 2008), but it can also be influenced by leaf structure and environmental factors (Fooshee *et al.* 1990, Užarević *et al.* 2011, Sheue *et al.* 2012). In *Arabidopsis* mutants, white leaf colour is mainly due to a defective chloroplast development (Sakamoto *et al.* 2009). However, Sheue (2012) proposed that white areas in some plants result from internal reflection between air spaces and leaf cells. Also, waxes or trichomes as well as the shape of epidermal cells affect plant colour (Reicosky and Hanover 1978, Kasperbauer and Wilkinson 1995, Glover and Martin 1998, Liakopoulos *et al.* 2006). In *A. kolomikta*, however, there were no trichomes or waxes. White areas of *A. kolomikta* leaves reflected more radiation than normal green areas. Many intercellular air spaces between the epidermal and palisade tissue layer or inside palisade parenchyma were observed in variegated leaves (Fig. 2). The intercellular air spaces were important factors in the formation of variegated leaves. During variegated leaf development, the content of anthocyanin and chlorophyll gradually increased. Hence, the formation of variegated leaves in *A. kolomikta* was strongly associated with the structure of the leaf interior and pigments.

Variegation in plants is usually thought to be the result of a single mechanism (Tsukaya *et al.* 2004, Merzlyak *et al.* 2008, Rocca *et al.* 2011, Sheue *et al.* 2012): either structural changes or a pigment content variation. However, we found that the leaf colour of *A. kolomikta* was influenced by the leaf structure, anthocyanin content, as well as chlorophyll content and distribution, which influenced optical properties of the leaf surface.

Under microscopic examination, white *A. kolomikta* leaves showed a distinct radiation reflection pattern that is different from that of *Begonia rex* Putz (Zhang *et al.*

reaching its highest value during the pink leaf period. In contrast, the abaxial ChlNDI, CRI, and ARI in variegated leaves were similar to those in green leaves.

2009, Sheue *et al.* 2012). White leaves of *A. kolomikta* were bright, and an epidermal cell shape did not vary significantly. It is unclear why white leaves in different plant species have different radiation reflection patterns. In *B. rex* and six other *Begonia* species, leaves have striking metallic patterns with variations of red and silver, but a large number of chloroplasts are still present in palisade tissue cells. Therefore, PP and SP are formed by the green background (palisade tissue cells). In white leaves of *A. kolomikta*, palisade tissue cells contained fewer chloroplasts, so the background was white. Thus, different reflectance patterns were observed under the different background.

Many studies document the use of leaf reflectance data to monitor plant growth and stress responses (Knippling 1970, Penuelas *et al.* 1997, Penuelas and Filella 1998, Sims and Gamon 2002, Bajgiran *et al.* 2012, Ranjan *et al.* 2014). Based on these reports, leaf reflection in visible wavelengths (400-700 nm) is determined by surface appendages (wax or trichomes) and photosynthetic pigments, but in near infrared (NIR) wavelengths (750 - 1350 nm), leaf reflection is primarily influenced by leaf structure (Slaton *et al.* 2001). Our results are consistent with studies showing that the NIR was significantly correlated with the leaf structure. In addition, reflectance is sensitive to changes of leaf colour; the reflectance curves, for example, were significantly different in the early pink and pink periods (Fig. 4). Leaf reflectance may have important applications. These include determination of leaf structure and leaf developmental stage, and estimation of photosynthetic potential of different plant species.

The results about the leaf optical properties and anatomy will help understanding the mechanism of variegation of leaves. We hope that the results of this study will stimulate other scientists to study variegated leaves of other plant species.

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