

Unique variegation characteristics of camellia cultivar 'Kumagai', in which chloroplasts disappear only in palisade tissue

Kento Terada

d21sc004@st.osaka-cu.ac.jp

Osaka City University: Osaka Koritsu Daigaku <https://orcid.org/0009-0002-7011-7581>

Chiyomi Uematsu

Osaka City University: Osaka Koritsu Daigaku

Toshihiro Yamada

Hokkaido University: Hokkaido Daigaku

Tomofumi Mochizuki

Osaka Metropolitan University: Osaka Koritsu Daigaku

Riichi Oguchi

Osaka Metropolitan University: Osaka Koritsu Daigaku

Research Article

Keywords: Camellia, Variegation, Plant viruses, Photosynthesis, Chloroplasts

Posted Date: November 10th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7964991/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Journal of Plant Research on August 10th, 2026. See the published version at <https://doi.org/10.1007/s10265-026-01740-1>.

1 Kento Terada
2 2000 Kisaichi, Katano-shi, Osaka-fu 576-0004, Japan
3 d21sc004@st.osaka-cu.ac.jp
4 Osaka City University
5 Osaka, Japan
6 Subject area: Ecology/Ecophysiology/Environmental Biology
7 Number of tables: 1
8 Number of figures: 10
9

Title

Unique variegation characteristics of camellia cultivar 'Kumagai', in which chloroplasts disappear only in palisade tissue.

Author

Kento Terada¹, Chiyomi Uematsu, Toshihiro Yamada², Tomofumi Mochizuki³, Riichi Oguchi³

¹Osaka City University, Osaka, Japan; ²Hokkaido University, Hokkaido, Japan; ³Osaka Metropolitan University, Osaka, Japan

Corresponding author: Kento Terada, d21sc004@st.osaka-cu.ac.jp

ORCID: 0009-0002-7011-7581, 0000-0002-9064-7048, 0000-0002-9129-4555, 0000-0003-1845-1558

Abstract

Variegation would be caused by reduction of chlorophyll and infection of pathogens, thus often decreases photosynthesis capacity. To elucidate the mechanisms to control the variegation, we observed an individual of camellia cultivar 'Kumagai' in which leaves are variegated only on the adaxial side. We found for the first time in woody seed plants that amount of chloroplasts was decreased only in palisade tissue while healthy chloroplasts were maintained in spongy tissue. Variegated leaves of 'Kumagai' maintained high Fv/Fm value in spite of lacking chloroplasts in palisade tissue. These results might suggest that healthy chloroplasts in spongy tissue mitigate the decrease in photosynthesis activity on variegated leaves. It should be also noted that highly variegated leaves were concentrated on specific branches. We also confirmed that this variegated 'Kumagai' was infected by Camellia japonica-associated betaflexivirus (CJaBV), camellia ringspot associated virus (CRSaV) and camellia yellow ringspot virus (CaYRV). These data might imply that unique variegated features of 'Kumagai' may be outcome of defense against viruses.

Keywords

Camellia, Variegation, Plant viruses, Photosynthesis, Chloroplasts

Introduction

Leaves or petals sometimes display zones of different colors, a phenomenon known as variegation. Variegation can result from anatomical mutations that alter light reflectance, such as increased air spaces in mesophyll tissue and specialized epidermal structures (Hara 1957). On the other hand, it may be caused by pathogens (Cheaib and Killiny 2024) or transposons (Tsugane et al. 2006), which influence pigment accumulation. Leaf variegation is observed in both herbaceous (Zhang et al. 2020a) and woody plants (Downton & Grant 1994), and classified into five types (Zhang et al. 2020b): Type I, chlorophyll type; Type II, air space type; Type III, epidermis type; Type IV, pigment type; and Type V, appendages type (cf. Hara 1957). Air space type is the most common, whereas chlorophyll type variegation is frequently seen in ornamental cultivars (Zhang et al. 2020a). Leaf variegation is generally expected to reduce photosynthetic productivity due to decreased chlorophyll content. Indeed, variegation of citrus leaves has been reported to inhibit plant growth (Xiong et al. 2019), which can lead economic losses in agriculture (Gonçalves et al. 2012). Conversely, variegated individuals are sometimes selectively propagated in horticulture for their ornamental value.

Native to East Asia, camellias (Theaceae) are now cultivated not only in Asia but also throughout Europe, the Americas, and other regions due to their economic and horticultural importance. They are used for tea production (*Camellia sinensis* (L.) Kuntze; Khan and Mukhtar 2013), oil (*C. oleifera* C. Abel; Quan et al. 2022), and as ornamental plants (*C. japonica* L. and related species; Valverde et al. 2012). Horticultural camellia varieties with variegated petals and leaves are highly valued for their ornamental beauty. Horticultural use of the variegation of camellia has a long history. Even in the 17th century, variegated camellias were already reported in Japanese horticultural books (Anrakuan 1630; Sakuma 2021). Various variegation patterns are recognized in Japanese cultivars of *C. japonica*, such as "tate-shibori" (a clear border along the main vein), and "yokomoku-shibori" (a random pattern without a clear border) (Kasahara 2008). The yokomoku-shibori pattern is thought to be caused by viruses, because the variegation trait can be transmitted by grafting, but is often not inherited through seed propagation (Kasahara 2008). In contrast, genetic factors such as transposons are considered as potential causes of the tate-shibori pattern. These variegation patterns often emerge along main vein of leaves and exhibit distinct margins. However, the specific genes, viruses, and mechanisms responsible for these patterns remain unclear.

In this study, we focus on the variegation of camellia variety ‘Kumagai’ (*C. japonica*), which exhibits varying degrees of leaf chlorosis: green, mottled, chlorotic, and senescent. Interestingly, in ‘Kumagai’, variegated sections of mottled and chlorotic leaves appear only on the adaxial side, while the abaxial side remains green; in senescent leaves, chlorosis occurs on both sides. These variations in variegation are a unique feature of “Kumagai”, and the effects of variegation on palisade versus spongy tissues have not been examined separately (Miura et al. 2010; Zhang et al. 2021). We investigated leaf anatomy, the viability of mesophyll cells and chloroplasts, and photosynthetic activities. In addition, we examined distribution of differently variegated leaves within a ‘Kumagai’ individual to infer how the variegation is transmitted within the plant.

Materials and methods

Sample preparation

Leaves were collected from a tree of *Camellia japonica* ‘Kumagai’ (ID 2F0083) cultivated in the Botanical Gardens, Osaka Metropolitan University. The extent of variegation varied among leaves on the tree.

Photographs of the leaves were taken alongside a black-and-white reference board. RGB values in the photographs were normalized using OpenCV-python (version 3.11.8) by comparing them to a reference image containing only the black-and-white board. Leaf areas were then cropped from each image.

To determine the threshold between variegated and the green areas, we generated a hue histogram based on images of five mottled leaves. For each image, pixel counts were normalized by the total number of pixels, and normalized counts were summed (Fig. 1). A valley appeared around a hue value of 70, separating the two peaks corresponding to the light green (variegated) and green (intact) regions. Based on this, a hue value of 70 was adopted as the threshold for variegation: leaf areas with hue value below 70 were considered variegated.

We measured the green and variegated areas to calculate the percentage of variegated area. When the variegated area was present only on the adaxial side, leaves were classified into three categories based on the percentage of variegated adaxial area: healthy green leaves (0-33%), mottled leaves (34-67%) and chlorotic leaves (68-100%). In the senescing leaves, where variegation appeared on both adaxial and

abaxial sides, adaxial variegation ranged from 68% to 100%, while abaxial variegation exceeded 40%. The average leaf longevity of *C. japonica* has been reported as 3.38 years (Nitta and Ohsawa 1997). To examine the distribution of variegated leaves within the tree, we analyzed only current-year leaves (emerged in 2022; <1 year old). All current-year leaves showed <40% variegation on the abaxial surface, which allowed us to distinguish them from senescing leaves. For all other analyses, we used the leaves older than one year.

Observation of leaf cross-section

To examine the distribution of chloroplasts in leaf tissue, leaves were sliced into 30 μ m sections by a linear slicer (Linear slicer pro 7, Dosaka EM, Kyoto, Japan). The sections were observed by a light microscope (BX50, Olympus, Tokyo, Japan). The leaf sections were also stained by 2 μ g/mL DAPI (4',6-diamidino-2-phenylindole) solution for 15 minutes. Stained sections were observed under fluorescence illumination using the same microscope. The excitation and emission peaks of DAPI are 360 nm and 460 nm, respectively.

Measurement of chlorophyll content

Two leaf disks (1 cm x 1cm) were excised from each leaf. One of the disks was separated into palisade and spongy tissues by a freezing microtome (MC-802A, KELK Ltd., Kanagawa, Japan). The separated tissues and the other intact leaf disk were each placed in 3 mL dimethylformamide (DMF) and kept at 4°C for one day. The absorbance of the DMF extracts was measured at 663.8 nm, 646.8 nm and 750 nm in a 10 mm quartz cuvette using a spectrophotometer (U-2810, HITACHI, Hitachi, Ltd., Tokyo, Japan) to calculate the chlorophyll content following the method of Porra et al. (1989).

Measurement of chlorophyll fluorescence

Prior to measuring the maximum photochemical efficiency of photosystem II (Fv/Fm), collected leaves were kept in the dark for 30 minutes to allow relaxation of non photochemical quenching. Fv/Fm was then measured using a chlorophyll fluorescence imaging system (FluorCam, Photon Systems Instruments, Průmyslová, Drásov, Czech Republic).

Fv/Fm and leaf color

To investigate the relationship between Fv/Fm and leaf color, we used two types of images. The first are images obtained by FluorCam system, which was used for measuring Fv/Fm. In these images, each pixel contains Fv/Fm information represented by color. The color information was converted to Fv/Fm value using a reference color bar, in which specific Fv/Fm values are correlated with the pixel colors. The resolution of the Fv/Fm values was 0.01.

The second is photographs taken with a digital camera (D5600, NIKON CORPORATION, Tokyo, Japan). The color information of these photographs was converted to hue values by “colorsys” module from OpenCV-python. The images were resized to match the pixel dimensions of the corresponding FluorCam images by “cv2.resize()” function. The aspect ratio of the second image was also adjusted to match the first image.

To align the position and tilt of two images, we used OpenCV-python to minimize the positional inconsistency between them. This was done by calculating the area of the non-overlapping regions when the two images were superimposed. The alignment was optimized by selecting the combination of rotation angle and position that resulted in the smallest gap area. Specifically, the angle and position of the second image were adjusted from -45 ° to 45 °, and from - leaf pixel size to + leaf pixel size in one-pixel increments, using the “cv2.rotate()” and “Numpy array slicing”. The leaf image analyzed consisted of ca. 30k pixels.

We extracted the overlapping pixels from both images to analyze the relationship between Fv/Fm values and hue values at each corresponding pixels. Scatter plots of hue versus Fv/Fm values were generated using “Scatter” function from plotly.graph_objects, and histograms of Fv/Fm values were created using “hist” function from matplotlib.pyplot.

For the analysis of the Fv/Fm within variegated areas, we excluded the border regions (with hue values larger than 65) and brownish areas (with hue values less than 60), which appeared to be caused by fungal infection. Accordingly, pixels with hue values between 60 and 65 were considered representative of variegated areas in this analysis.

Distribution of differently variegated leaves in a tree

The percentage of variegated area were calculated for the all leaves less than one-year old, as described

above. Branching points were designated as B1 to B18 from the base to the top of the plant.

To examine whether highly variegated leaves are concentrated within specific branch systems, we performed the following statistical tests at each branching point: (1) Fisher's exact test was conducted on the two daughter branches diverging at each branching point with classifying the leaves into chlorosis or not; (2) Permutation test (Good 2005) was applied using the variance reduction in percentage of variegated area (Online Resource 2) which was calculated by splitting at the branching point (Breiman et al. 1984; Chen and He 2019); (3) Permutation test using information gain was conducted based on entropy calculations (Quinlan 1986; Raileanu and Stoffel 2004). In this test, leaves were classified as green, mottled, or chlorotic. All calculations were performed by using Python : "fisher_exact()" from scipy.stats, for the Fisher's exact test; "numpy.var()" to compute calculate variance; "entropy" from scipy.stats for calculating entropy.

Virus identification

Viruses potentially infecting "Kumagai" were identified using Next-generation Sequencing (NGS) (Hadidi et al. 2016). For virus identification, 2 cm by 2 cm square pieces of fresh mottled leaf were collected from several leaves. From these samples, total RNA was extracted by a modified CTAB method (Li et al. 2008) and purified with spin columns of RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with RNase-Free DNase (Qiagen). The concentration and purity of the extracted RNA were assessed by BioPhotometer D30 (Eppendorf, Hamburg, Germany). RNA libraries were prepared with "TruSeqStranded Total RNA with Ribo-Zero" kit (Illumina, San Diego, California, U.S.) following ribosomal RNA removal. Sequencing was conducted on Illumina NovaSeq 6000 system (Illumina) with 100 bp paired-end reads.

Adapter sequences, low-quality sequences and ambiguous bases (N) were trimmed by Trimmomatic (Bolger et al. 2014). The cleaned reads were *de novo* assembled by Trinity (Grabherr et al. 2011). To identify viral sequence, contigs were subjected to BLASTN and BLASTX searches viruses database (taxid: 10239) of the National Center for Biotechnology Information. A virus was considered present in 'Kumagai' if a contig exhibited a significant match to a viral reference in both BLASTN and BLASTX (e-value < 1e-10), with $\geq 80\%$ coverage, $\geq 90\%$ identity, and a length exceeding 1,000 bp. RNA-Seq data analysis was performed on the supercomputer available in National Institute of Genetics.

Result

Distribution of chloroplasts in variegated leaves

Chloroplasts were not observed in the epidermal or vascular tissues in any variegation examined (Fig. 2). In green leaves, chloroplasts were present in mesophyll tissue, although they were sparsely distributed in spongy parenchymatous cells (Fig. 2a, b). In mottled leaves, the number of chloroplasts was reduced in palisade parenchyma cells of the variegated region (Fig. 2c, d). In chlorotic leaves, chloroplasts were rarely observed in palisade parenchyma cells, although they were present in cells adjacent to vascular cylinder (Fig. 2e, f). Notably, the number of chloroplasts in the spongy parenchyma cells did not differ between the variegated and healthy green leaves. In some samples, chloroplasts were even more densely concentrated in the spongy parenchyma cells, especially where the adjacent palisade parenchyma cells contained few or no chloroplasts. In senescing leaves, chloroplasts were scarce in both palisade and spongy tissues (Fig. 2g, h).

Nuclear viability in variegated leaves

To assess the viability of the chlorotic cells, the appearance of nucleuses in palisade parenchyma cells was compared between green and weakly chlorotic leaves using DAPI staining (Fig.3). In green leaves, chlorophyll fluorescence from chloroplasts was observed both in palisade and spongy parenchyma cells. In chlorotic leaf, the intensity of chloroplast fluorescence in palisade cells was clearly lower than that in the green leaves. However, despite the markedly reduced fluorescence from chloroplasts, the shape and DAPI fluorescence intensity of the nuclei were similar between green and chlorotic leaves. Therefore, we concluded that the nuclei in the palisade cells of the variegated section remained in a healthy condition.

Chlorophyll content in variegated leaves

To examine how the number of chloroplasts changes in palisade and spongy tissues of the variegated sections, we compared the chlorophyll content among healthy, mottled, chlorotic, and senescing leaves. In the palisade tissue of chlorotic leaves, chlorophyll content was significantly lower than that of green leaves (Fig. 4a), while no significant difference was observed in the spongy tissue (Fig. 4b). Accordingly,

the whole-tissue analysis including palisade and spongy tissues showed that the chlorophyll content of chlorotic leaves was significantly lower than that of green and mottled leaves (Fig. 4c). In senescing leaves, chlorophyll content was significantly lower than that of green leaves in both palisade and spongy tissues, as well as in the whole-tissue analysis.

To compare the extent of chlorophyll reduction between palisade and spongy tissues of variegated leaves, relative chlorophyll contents were calculated for each type with respect to healthy green leaves (Fig. 4d). In chlorotic leaves, the chlorophyll a + b content in palisade tissue was only 20% of that in green leaves, whereas in the spongy tissue, it was 60%. Thus, the reduction in chlorophyll content was smaller in spongy tissue than in palisade tissue.

Photosynthetic activity in variegated leaves based on Fv/Fm values

As shown in previous studies, healthy leaves typically exhibit Fv/Fm values around 0.8 (Björkman and Demmig 1987). Therefore, we used a two-dimensional chlorophyll fluorometer to measure Fv/Fm values and assessed the photosynthetic activity in green and variegated leaves (Fig. 5). The mean Fv/Fm values for green, mottled, chlorotic and senescing leaves were 0.78, 0.77, 0.79 and 0.52, respectively (Fig. 6). No significant differences were observed between the adaxial and abaxial sides across the four leaf types.

In green leaves, the hue value were mostly concentrated around 100. In mottled leaves, they ranged from 60 to 100, and in chlorotic leaves, from 55 to 90. In senescing leaves, they were concentrated around 60, and at most around 80. The Fv/Fm values of green, mottled, and chlorotic leaves were distributed with a peak at around 0.8, while senescing ones showed around 0.7.

In all types, the correlations between Fv/Fm values and leaf color were very weak ($R^2 < 0.09$; Fig. 7e–h), although *P*-values were low due to the large number of pixels analyzed (more than 40000). However, it should be noted that Fv/Fm values were consistently lower at the brown colored spots, which appeared to be colonized by fungi (Fig. 7d, h).

In mottled and chlorotic leaves, variegated sections were rarely observed on abaxial side (Fig. 5e–g), whereas in senescing leaves, variegated sections were visible even on the abaxial side (Fig. 5h). In some cases, chlorotic sections extended across both adaxial and abaxial tissues in senescing leaves (Fig. 5d, h). In such case, Fv/Fm values were too low to be detected, as indicated by the white pixels in Fig. 5d and h.

There were no significant differences in the mean Fv/Fm values among green, mottled and chlorotic

leaves (Fig. 6). However, the mean Fv/Fm value of senescing leaves was significantly lower than those of the other leaf types on both adaxial and abaxial sides. When analysis was restrictedly conducted on variegated sections excluding the boundary regions between variegated and green sections, the Fv/Fm values in variegated sections of mottled leaves were significantly lower than that of green and chlorotic leaves (Fig. 8).

Relationships among the percentage of variegated area, chlorophyll content, and Fv/Fm value

When data of the four types of leaves were analyzed, a negative correlation was observed between the percentage of variegated area and chlorophyll content (Fig. 9a). This negative correlation persisted even when senescing leaf samples were excluded. In the scatter plot of chlorophyll content versus Fv/Fm value, a positive correlation was observed only when senescing leaves were included (Fig. 9b). Similarly, in the scatter plot of the percentage of variegated area versus Fv/Fm value, a negative correlation was evident only when senescing leaves were included (Fig. 9c). These results suggest that photosynthetic activity of chloroplasts was not diminished in mottled leaves and chlorotic leaves.

Distributions of variegated leaves in tree

When leaves were divided into two groups at B1 or B3 branching point (in the case of the analysis at B3 branching point, leaves with the number smaller than 28 were not included), significant difference in variegation dispersion were observed at all the following three tests: Fisher's exact test, variance reduction and information gain (Online Resource 1). Therefore, we considered that distribution of the chlorosis type of leaves was concentrated on some branches. It would be also able to suggest that leaves on adjacent branches had similar ratio of variegated area.

Possibility of virus infection on 'Kumagai'

NGS analysis of RNA libraries from mottled leaves of "Kumagai" yielded 50,793 contigs potentially derived from viruses. Among these, 16 contigs, ranging 1,219 to 7,834 bp in length, showed coverage and identity values of 88.2 to 100 % and 90.0 to 98.7 %, respectively, against the genome and protein of three known camellia viruses. These values exceeded the threshold criteria for confirming viral infection. The identified viruses were: camellia ringspot-associated virus (CRSaV) (Liu et al. 2019; Zheng et al. 2020),

Camellia japonica-associated betaflexivirus (CJaBV) (Peracchio et al. 2020) and camellia yellow ringspot virus (CaYRV) (Zhang et al. 2020b). For each virus, we listed one contig that showed the highest sequence identity to both its genome and protein in Table 1.

Discussion

Dorsiventral disappearance of chloroplasts in leaves in ‘Kumagai’

In variegated sections of chlorotic leaves, chloroplasts were absent in the palisade parenchyma cells, while their number did not decrease and sometimes even increased in the spongy parenchyma cells (Fig. 2-3). This observation is supported by the lower chlorophyll content in the palisade tissue of chlorotic leaves compared to healthy green leaves (Fig. 4a), while the chlorophyll content in the spongy tissue showed no significant difference between chlorotic and green leaves (Fig. 4b). The adaxial surface color clearly differed between chlorotic and green leaves, whereas the abaxial surface color appeared similar in the two types (Fig. 5). Additionally, neither cell necrosis nor nuclear collapse was observed in the variegated sections of chlorotic leaves (Fig. 3).

These results suggested that chlorotic symptom in ‘Kumagai’ are caused by a reduction in the chloroplast number specifically in palisade tissue, a phenomenon consistent with chlorophyll-type variegation *sensu* (Zhang et al. 2020a). However, such chloroplast loss restricted to the palisade tissue has not previously been reported in woody plants. Although ‘Kumagai’ lacks specialized epidermis nor air spaces, the chloroplast-deficient palisade tissue may contribute to alter light refraction, reflection and scattering, similar to the role played by air spaces or specialized epidermal cells in other types of variegation.

Uniqueness of the chlorophyll-type variegation found in ‘Kumagai’

It has been reported that Fv/Fm values decrease in the variegated sections of chlorophyll-type variegation. For example, *Ficus pumila* L. ‘Sonny’ exhibits chlorophyll-type variegation, in which Fv/Fm value of white section (0.13) is significantly lower than green section (0.83) (Sheue et al. 2012). In contrast, variegation in begonia is categorized as air space-type, caused by intercellular spaces above the chlorenchyma (Sheue et al. 2012). In such cases, photosynthetic activity does not differ between white and green sections because cellular structure and contents remain unchanged.

As discussed above, 'Kumagai' exhibits chlorophyll-type variegation. However, the Fv/Fm values of mottled and chlorotic leaves were not significantly different from those of green leaves on both adaxial and abaxial sides (Fig. 6). In 'Kumagai', although the number of chloroplast are reduced in the palisade tissue of the variegated sections, this reduction is minimized at the whole-leaf level because the number of chloroplasts in the spongy tissue may remain unchanged or even increase. These changes may help compensate for the reduced photosynthetic capacity in the palisade tissue, thereby maintaining the overall photosynthetic activity of the leaf.

Blastus cochinchinesis Lour. is also known to possess few chloroplasts in the adaxial mesophyll of white sections of variegated leaves (Chen et al. 2017), suggesting a potentially similar variegation mechanism to that of 'Kumagai'. However, notable differences exist between the two species: in the variegated section of 'Kumagai', the palisade and spongy tissues are clearly differentiated, whereas in the white sections of *B. cochinchinesis*, the entire mesophyll consists of parenchyma cells which are similar to those constituting the spongy tissue in non-variegated sections. Therefore, the variegation in *B. cochinchinesis* can be categorized not only as chlorophyll-type but also as variable palisade-type variegation (La Rocca et al. 2011). It should also be noted that the variegation in *B. cochinchinesis* is not virus-induced, but represents an ontogenetic trait: the variegation appears only in leaves that develop during the juvenile stage when the plant height is less than 15 cm (Chen et al. 2017).

Changes in photosynthetic activity along the chlorosis gradient

When the boundary regions between variegated and green sections (hue value 65–70) were excluded from analysis, the Fv/Fm values of the interior portions of variegated sections (hue value 60–65) were significantly lower in mottled leaves than in green and chlorotic leaves (Fig. 8). This lower Fv/Fm value may indicate the onset of chloroplast decomposition in the palisade tissue of mottled leaves. Reduced photosynthetic activity in such tissue is commonly associated with virus-induced damage. For example, viral infections are known to deform chloroplast structure and decreases Fv/Fm values in cassava plants (Zanini et al. 2021).

It has also been reported that damaged chloroplasts can be digested via autophagy (chlorophagy) (Nakamura et al. 2018). Therefore, the disappearance of the chloroplasts in the palisade tissue of the variegated sections in mottled and chlorotic leaves may result from autophagic degradation of

dysfunctional chloroplasts. On the other hand, the larger number of chloroplasts observed in parts of the spongy tissue of chlorotic leaves, compared to green leaves, may indicate chloroplasts were synthesized *de novo* in the spongy tissue following the chloroplast degradation in the palisade tissue (Fig. 2).

In senescing leaves of 'Kumagai', few chloroplasts remain in both palisade and spongy tissues and Fv/Fm values are low. This trait was not observed in mottled or chlorotic leaves, suggesting that the cause of chlorosis in senescing leaves differs from that in mottled and chlorotic leaves. Various pathways for chloroplast degradation during leaf senescence have been reported (Izumi and Nakamura 2018; Domínguez and Cejudo 2021) , and in senescing leaves of “Kumagai”, chloroplast degradation may represent a preparatory process for leaf abscission.

Variegation potentially induced by virus in ‘Kumagai’

Chlorotic symptoms in plants can sometimes be caused by pathogenic viruses or associated physiological disorders (Cheaib and Killiny 2024). In ‘Kumagai’, the infecting viruses were identified by NGS analysis (Table 1). In this 'Kumagai', the observed symptoms differ from known those typically associated with CRSaV, CJaBV or CaYRV. This discrepancy may reflect differences in host genetic background and/or the multiple viral infections (Zhang et al. 2020b). Because camellias have been propagated by grafting for ornamental use, coinfection likely occurs (Liu et al. 2019). Thus, both multiple viral infection and host genetic background may contribute to variegation in camellia cultivars.

In the virus-induced variegation, it is expected that virus-infected cells increase more rapidly in tissues with densely packed cells, as close cell-to-cell contact facilitates virus spread. Our observation supports this hypothesis: chloroplast reduction was more pronounced in the palisade tissue than in the spongy tissue, consistent with denser cellular arrangement in the former (Figs. 2 and 3).

We also observed that severely variegated leaves in “Kumagai” were restricted to specific branches (Fig. 10). This may suggest that *Camellia japonica* possesses a defense mechanism that limit viral spread between branches, thereby maintaining overall photosynthetic activity. It is known that, under certain conditions, plants can suppress symptom development and recover from viral infection (Ghoshal and Sanfaçon 2015). For example, replication and movement of melon necrotic spot virus (MNSV) are restricted at temperatures above 25°C (Kido et al. 2008). Whether a similar defense mechanism to repress viral replication operates in *C. japonica* warrants further investigation.

Conclusion

In this study, we revealed that variegation of ‘Kumagai’ was caused by the decrease in the amount of chloroplasts in palisade tissue: ‘chlorophyll’ type variegation. However, 'Kumagai' maintains high Fv/Fm value by keeping or sometimes even increasing the amount of chloroplasts in spongy tissue, which may help maintain photosynthesis activity.

Acknowledgements

This work was supported by JST, the establishment of university fellowships towards the creation of science technology innovation (JPMJFS2138). This work was also supported by MEXT Promotion of Distinctive Joint Usage/Research Center Support Program (JPMXP0723833155). Computations were partially performed on the NIG supercomputer at ROIS National Institute of Genetics.

Declarations

Funding

This study was partly supported by the following grants: JST Grant “Establishment of University Fellowships toward the Creation of Science and Technology Innovation” (JPMJFS2138), and the MEXT Promotion of Distinctive Joint Usage/Research Center Support Program (JPMXP0723833155).

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

References

- Anrakuan S (1630) Hyakuchinshuu
- Björkman O, Demmig B (1987) Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170:489–504. <https://doi.org/10.1007/BF00402983>
- Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Breiman L, Friedman JH, Olshen RA, Stone CJ (1984) Classification And Regression Trees, 1st edn. Chapman and Hall/CRC
- Cheaib A, Killiny N (2024) Photosynthesis Responses to the Infection with Plant Pathogens. *Mol Plant*

Microbe Interact. <https://doi.org/10.1094/MPMI-05-24-0052-CR>

Chen E, He XJ (2019) Crude Oil Price Prediction with Decision Tree Based Regression Approach. *Journal of International Technology and Information Management* 27:2–16. <https://doi.org/10.58729/1941-6679.1391>

Chen YS, Chesson P, Wu HW, et al (2017) Leaf structure affects a plant's appearance: combined multiple-mechanisms intensify remarkable foliar variegation. *J Plant Res* 130:311–325. <https://doi.org/10.1007/s10265-016-0890-4>

Domínguez F, Cejudo FJ (2021) Chloroplast dismantling in leaf senescence. *J Exp Bot* 72:5905–5918

Downton W, Grant W (1994) Photosynthetic and Growth Responses of Variegated Ornamental Species to Elevated CO₂. *Functional Plant Biology* 21:273–279. <https://doi.org/10.1071/PP9940273>

Ghoshal B, Sanfaçon H (2015) Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. *Virology* 479–480:167–179

Gonçalves FP, Stuchi ES, Lourenço SA, et al (2012) Relationship between sweet orange yield and intensity of Citrus Variegated Chlorosis. *Plant Pathol* 61:641–647. <https://doi.org/10.1111/j.1365-3059.2011.02557.x>

Good P (2005) *Permutation, Parametric and Bootstrap Tests of Hypotheses*, 3rd edn. Springer-Verlag, New York

Grabherr MG, Haas BJ, Yassour M, et al (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol* 29:644–652. <https://doi.org/10.1038/nbt.1883>

Hadidi A, Flores R, Candresse T, Barba M (2016) Next-generation sequencing and genome editing in plant virology. *Front Microbiol* 7:. <https://doi.org/10.3389/fmicb.2016.01325>

Hara N (1957) Study of the variegated leaves, with special reference to those caused by air spaces

Izumi M, Nakamura S (2018) Chloroplast protein turnover: The influence of extraplastidic processes, including autophagy. *Int J Mol Sci* 19

Kasahara K (2008) The story of the spotted plant. Why do different flowers bloom from one tree?

Khan N, Mukhtar H (2013) Tea and health: studies in humans. *Curr Pharm Des* 19:6141–7. <https://doi.org/10.2174/1381612811319340008>

Kido K, Tanaka C, Mochizuki T, et al (2008) High temperatures activate local viral multiplication and cell-to-cell movement of Melon necrotic spot virus but restrict expression of systemic symptoms. *Phytopathology* 98:181–186. <https://doi.org/10.1094/PHYTO-98-2-0181>

La Rocca N, Rascio N, Pupillo P (2011) Variegation in *Arum italicum* leaves. A structural-functional study. *Plant Physiology and Biochemistry* 49:1392–1398. <https://doi.org/10.1016/j.plaphy.2011.09.009>

Li R, Mock R, Huang Q, et al (2008) A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens. *J Virol Methods* 154:48–55. <https://doi.org/10.1016/j.jviromet.2008.09.008>

- Liu H, Wu L, Zheng L, et al (2019) Characterization of three new viruses of the family Betaflexiviridae associated with camellia ringspot disease. *Virus Res* 272:.. <https://doi.org/10.1016/j.virusres.2019.197668>
- Miura E, Kato Y, Sakamoto W (2010) Comparative transcriptome analysis of green/white variegated sectors in *Arabidopsis* yellow variegated2: Responses to oxidative and other stresses in white sectors. *J Exp Bot* 61:2433–2445. <https://doi.org/10.1093/jxb/erq075>
- Nakamura S, Hidema J, Sakamoto W, et al (2018) Selective elimination of membrane-damaged chloroplasts via microautophagy. *Plant Physiol* 177:1007–1026. <https://doi.org/10.1104/pp.18.00444>
- Nitta I, Ohsawa M (1997) Leaf dynamics and shoot phenology of eleven warm-temperate evergreen broad-leaved trees near their northern limit in central Japan. Kluwer Academic Publishers
- Peracchio C, Forgia M, Chiapello M, et al (2020) A complex virome including two distinct emaraviruses associated with virus-like symptoms in *Camellia japonica*. *Virus Res* 286:.. <https://doi.org/10.1016/j.virusres.2020.197964>
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 975:384–394. [https://doi.org/https://doi.org/10.1016/S0005-2728\(89\)80347-0](https://doi.org/https://doi.org/10.1016/S0005-2728(89)80347-0)
- Quan W, Wang A, Gao C, Li C (2022) Applications of Chinese *Camellia oleifera* and its By-Products: A Review. *Front Chem* 10
- Quinlan JR (1986) Induction of decision trees. *Mach Learn* 1:81–106. <https://doi.org/10.1007/BF00116251>
- Raileanu LE, Stoffel K (2004) Theoretical comparison between the Gini Index and Information Gain criteria *. Kluwer Academic Publishers
- Sakuma D (2021) A list of Dr. Shin-ichiro Kishikawa's "Kishikawa Camellia Book Collection." Osaka Museum of Natural History, Osaka
- Sheue CR, Pao SH, Chien LF, et al (2012) Natural foliar variegation without costs? the case of *Begonia*. *Ann Bot* 109:1065–1074. <https://doi.org/10.1093/aob/mcs025>
- Tsugane K, Maekawa M, Takagi K, et al (2006) An active DNA transposon nDart causing leaf variegation and mutable dwarfism and its related elements in rice. *Plant Journal* 45:46–57. <https://doi.org/10.1111/j.1365-313X.2005.02600.x>
- Valverde RA, Sabanadzovic S, Hammond J (2012) Viruses that enhance the aesthetics of some ornamental plants: Beauty or beast? *Plant Dis* 96:600–611. <https://doi.org/10.1094/PDIS-11-11-0928-FE>
- Xiong B, Qiu X, Huang S, et al (2019) Physiological and transcriptome analyses of photosynthesis

and chlorophyll metabolism in variegated Citrus (Shiranuhi and Huangguogan) seedlings. Sci Rep 9:. <https://doi.org/10.1038/s41598-019-52276-5>

Zanini AA, Di Feo L, Luna DF, et al (2021) Cassava common mosaic virus infection causes alterations in chloroplast ultrastructure, function, and carbohydrate metabolism of cassava plants. Plant Pathol 70:195–205. <https://doi.org/10.1111/ppa.13272>

Zhang JH, Zeng JC, Wang XM, et al (2020a) A revised classification of leaf variegation types. Flora: Morphology, Distribution, Functional Ecology of Plants 272:. <https://doi.org/10.1016/j.flora.2020.151703>

Zhang Q, Huang J, Zhou P, et al (2021) Cytological and transcriptomic analysis provide insights into the formation of variegated leaves in *Ilex × altaclerensis* ‘belgica aurea.’ Plants 10:1–17. <https://doi.org/10.3390/plants10030552>

Zhang S, Yang L, Ma L, et al (2020b) Virome of *Camellia japonica*: Discovery of and Molecular Characterization of New Viruses of Different Taxa in Camellias. Front Microbiol 11:. <https://doi.org/10.3389/fmicb.2020.00945>

Zheng L, Chen M, Li R (2020) *Camellia* ringspot-associated virus 4, a proposed new foveavirus from *Camellia japonica*. Arch Virol 165:1707–1710. <https://doi.org/10.1007/s00705-020-04655-x>

472 Table 1. BLAST results of virus-like contigs

	Contig ID	Contig length	Subject Seq ID	Subject Seq length	Identity (%)	Number of identity	Number of mismatches	Number of gaps	Virus
BLASTN	534247	6040	MN532566.1	7791	96.507	5829	211	0	CJaBV
	539362	5406	MT028514.1	8829	96.818	5233	172	0	CRSaV
	48137	5282	MT036046.1	5322	95.152	5024	256	0	CaYRV
BLASTX	534247	6040	QGY72525.1	1990	97.617	1393	34	0	CJaBV
	539662	7784	QEJ80625.1	1991	91.311	1818	173	0	CRSaV
	48137	5282	QID58999.1	1728	98.322	1699	29	0	CaYRV

473 CJaBV: *Camellia japonica* associated betaflexivirus; CRSaV: camellia ringspot associated virus; CaYRV: camellia yellow ringspot virus

474

Figure legends

Fig. 1 Relative frequency of pixel hue in photographs of the adaxial side of mottled leaves. Class interval was two (Bin = 2)

Fig. 2 Photographs of healthy green leaf (a), mottled leaf (c), chlorotic leaf (e) and senescing leaf (g) of *Camellia japonica* 'Kumagai'. All the leaves were older than one year old. (b), (d), (f) and (h) show the cross-sectional photographs of (a), (c), (e) and (g), respectively. In (d) and (f), the cross section was gained from variegated section. In (a, c, e, g), scale bar means 5cm, and in (b, d, f, h), scale bar means 100 μ m

Fig. 3 Photographs of the cross section of green leaf (a) and chlorotic leaf (b) stained by DAPI. Red fluorescence is derived from chlorophyll in chloroplasts, and blue fluorescence is derived from nucleus. The scale bar is 100 μ m

Fig. 4 Chlorophyll content per leaf area in palisade tissue (a), in spongy tissue (b) and in whole tissue (c) are shown. Relative chlorophyll contents of each leaf compared to the green leaves are shown (d). All data are presented as Mean $\pm$ SD. Different alphabets indicate statistically significant differences among different chlorotic leaves

Fig. 5 In each panel upper side is a photograph of a leaf and lower side is an image of measured Fv/Fm value. Adaxial side (a–d) and abaxial side (e–h) of green- (a, e), mottled- (b, f), chlorotic- (c, g) and senescing-leaf (d, h) were shown. The scale bar is 5 cm. The color bar indicates Fv/Fm value from 0.65 to 0.85. In senescing leaves, Fv/Fm values of chlorotic section in both sides were too low and could not be detected. In senescing leaf, there was no Fv/Fm value in the area which shows chlorosis on both sides of the leaf. In such an area, color for the Fv/Fm value was shown by white. Photographs and two-dimension images of Fv/Fm value data of abaxial side were flipped reversed up and bottom for easy comparison with adaxial side

Fig. 6 Fv/Fm value of adaxial and abaxial side of the four types of leaves. Mean $\pm$ SD is shown. Different alphabets indicate the significant differences among different chlorotic leaves

Fig. 7 A photograph of leaves (a–d). A scatter plot between hue value and Fv/Fm value of each pixel (e–h). The color of each plot indicates each pixel color.

Regressions (e), $R^2 = 0.0857$; (f), $R^2 = 0.0281$; (g), $R^2 = 0.0136$; (h), $R^2 = 0.0307$

Fig. 8 Fv/Fm value of the inside of variegated section (hue value 60-65) in the four types of leaves. Mean $\pm$ SD is shown. Different alphabets indicate the significant difference among different chlorotic leaves

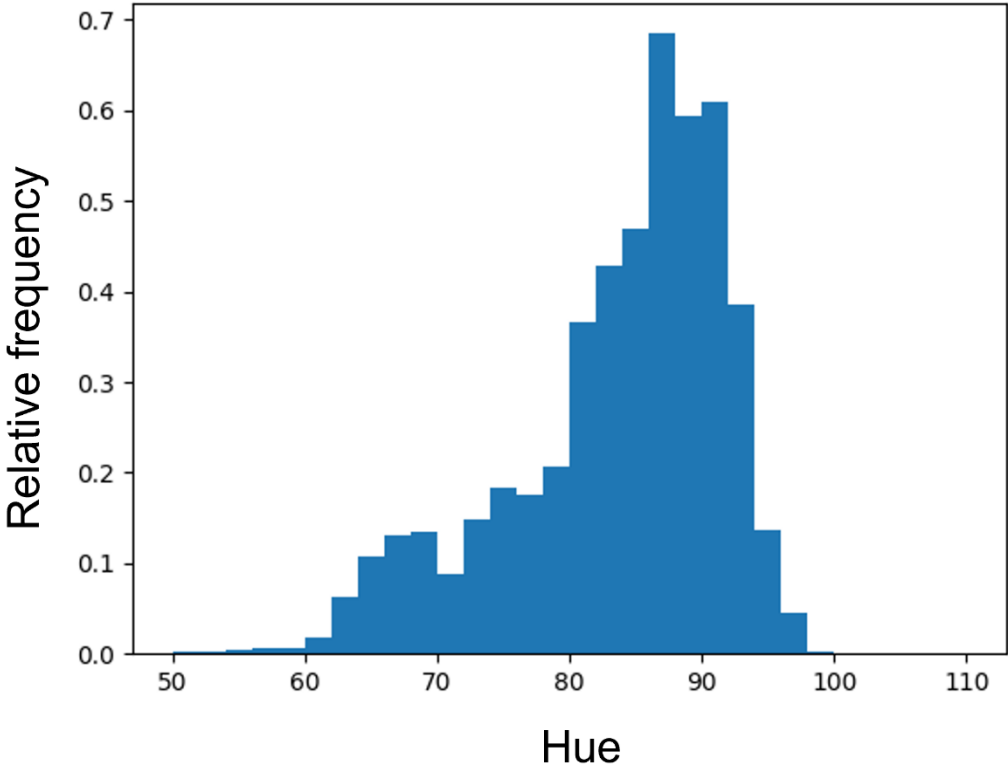
Fig. 9 Scatter plots between the percentage of variegated area and chlorophyll content (a), between

chlorophyll content and Fv/Fm value **(b)**, and between the percentage of variegated area and Fv/Fm value **(c)** were shown. The color of each plot indicates the type of leaf classified by the percentage of variegated area. Solid and dotted lines are regression lines of all leaf types and without senescing leaf type respectively.

Regressions **(a)**, all: $Y = 23.559 - 0.1644X$, $R^2 = 0.8583$, $P\text{-value} < 0.001$, without senescing: $Y = 23.008 - 0.11304X$, $R^2 = 0.8546$, $P\text{-value} < 0.001$; **(b)**, all: $Y = 0.4596 + 0.0152X$, $R^2 = 0.4226$, $P\text{-value} = 0.022$, without senescing: $Y = 0.7987 - 0.0019X$, $R^2 = 0.2208$, $P\text{-value} = 0.202$; **(c)**, all: $Y = 0.7893 - 0.002X$, $R^2 = 0.2368$, $P\text{-value} = 0.109$, without senescing: $Y = 0.7531 + 0.0003X$, $R^2 = 0.2559$, $P\text{-value} = 0.165$

Fig. 10 Circles and lines mean leaves and branches of ‘Kumagai’. Green lines show petioles, and black lines show branches. Colors of circles indicate a type of variegation; green, yellow and white indicate green-, mottled- and chlorotic-leaves, respectively. The length of the lines does not indicate the distance among leaves. They only show the branching patterns. Branching points with blue circle indicate points where significant differences in variegation dispersion were observed (Online Resource 1)

521

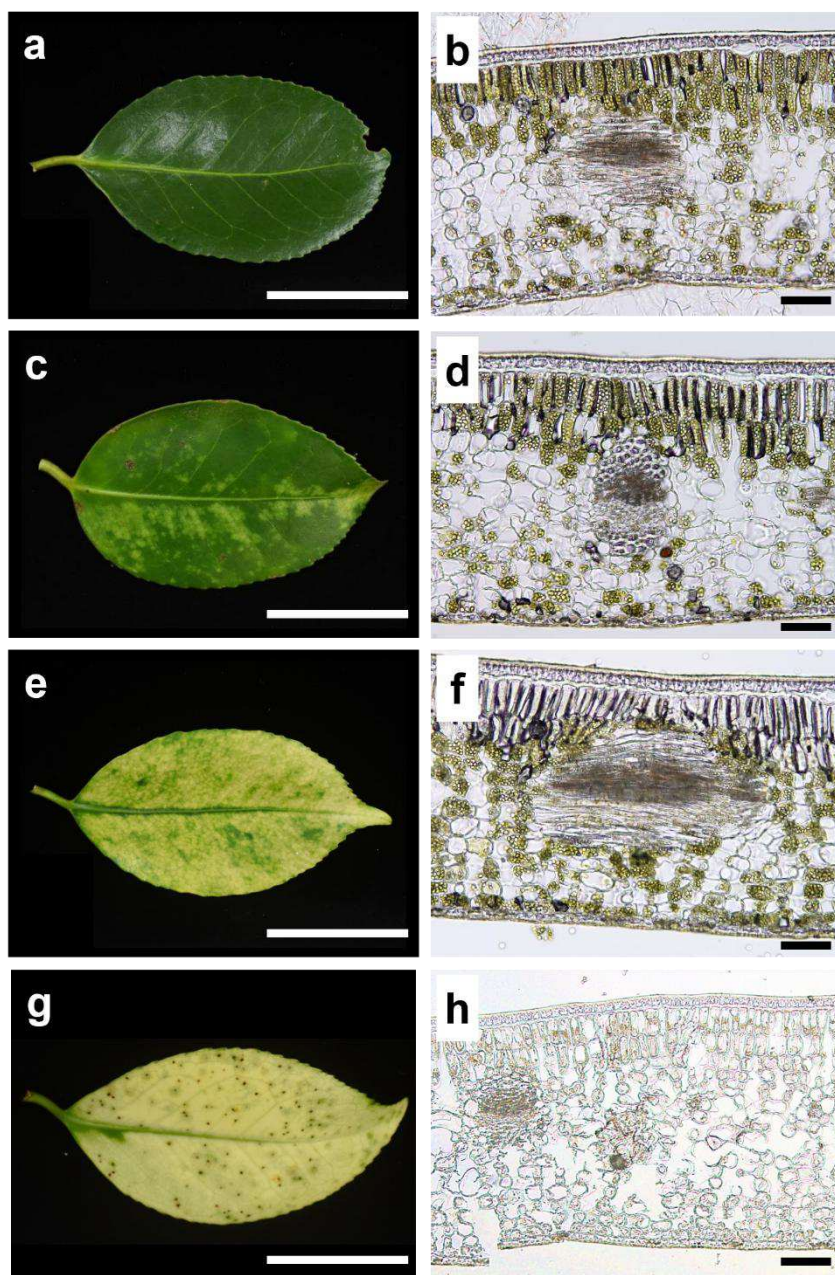


522

523 **Fig. 1** Relative frequency of pixel hue in photographs of the adaxial side of mottled leaves. Class interval
524 was two (Bin = 2)

525

526



Scale bar: 5 cm

Scale bar: 100 μ m

Fig. 2 Photographs of healthy green leaf (a), mottled leaf (c), chlorotic leaf (e) and senescing leaf (g) of *Camellia japonica* 'Kumagai'. All the leaves were older than one year old. (b), (d), (f) and (h) show the cross-sectional photographs of (a), (c), (e) and (g), respectively. In (d) and (f), the cross section was gained from variegated section. In (a, c, e, g), scale bar means 5cm, and in (b, d, f, h), scale bar means 100 μ m

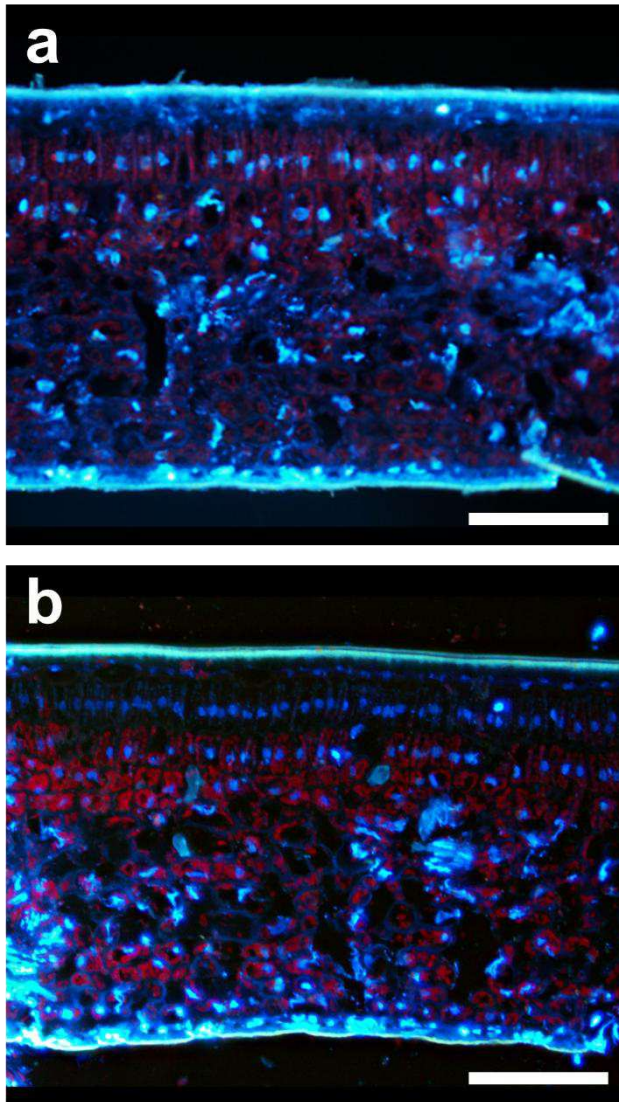


Fig. 3 Photographs of the cross section of green leaf (**a**) and chlorotic leaf (**b**) stained by DAPI. Red fluorescence is derived from chlorophyll in chloroplasts, and blue fluorescence is derived from nucleus. The scale bar is 100 μm

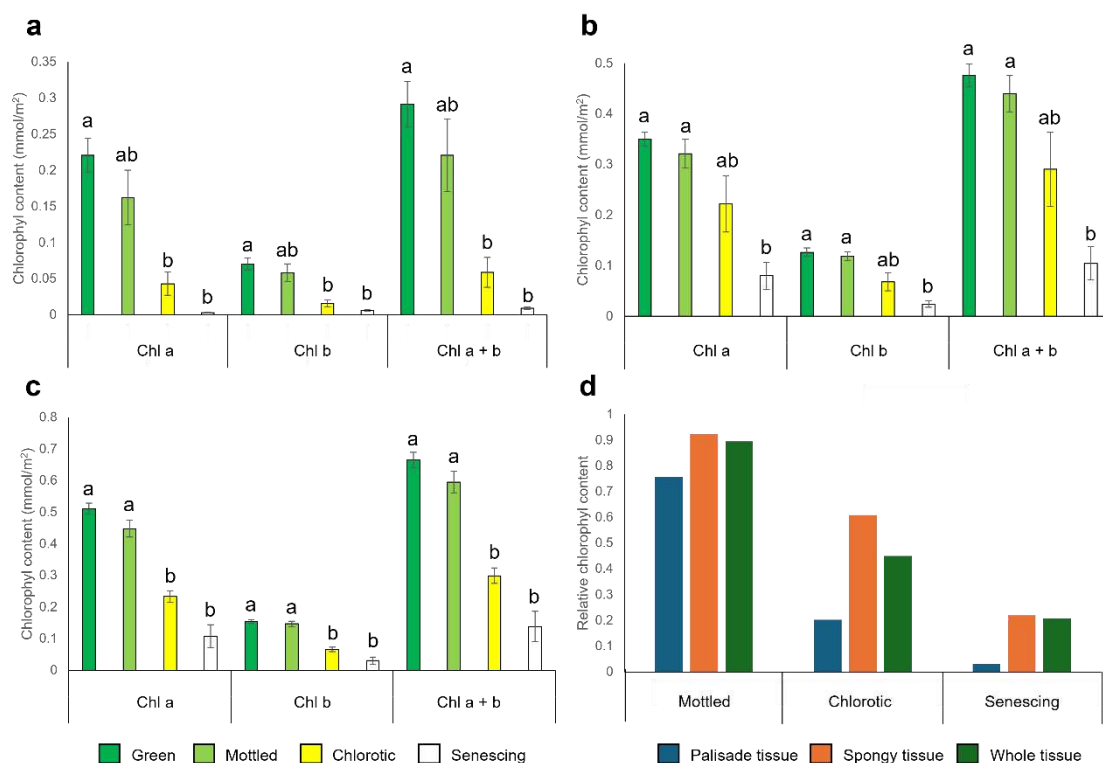
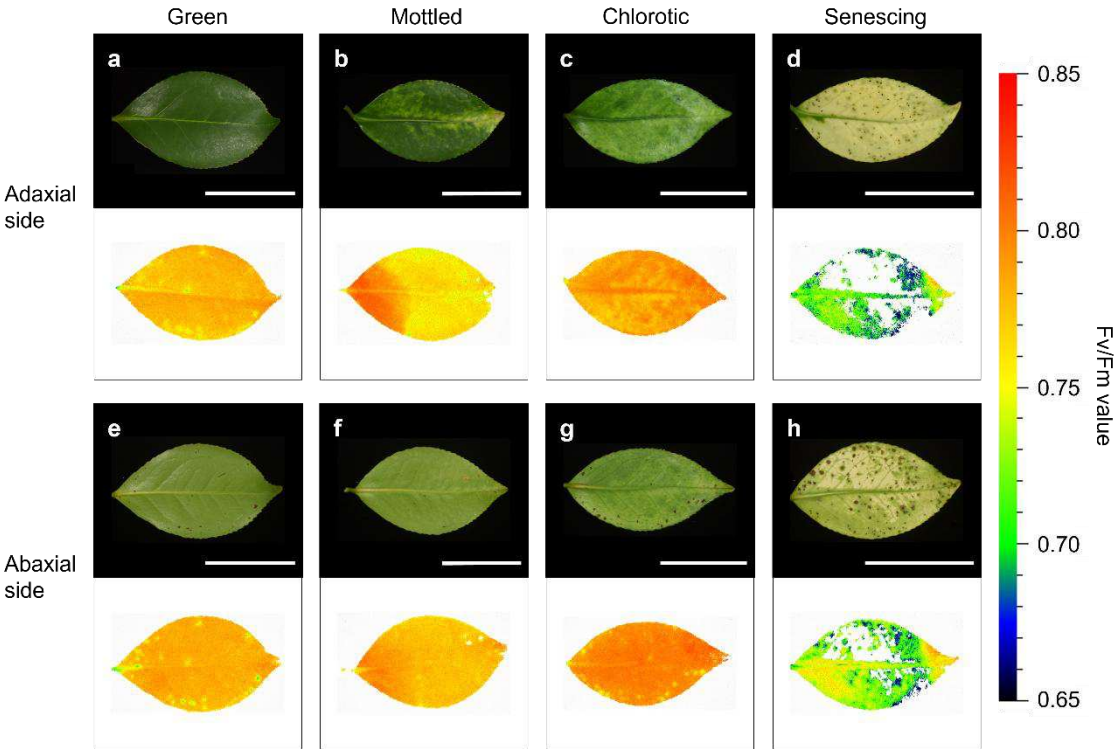


Fig. 4 Chlorophyll content per leaf area in palisade tissue (a), in spongy tissue (b) and in whole tissue (c) are shown. Relative chlorophyll contents of each leaf compared to the green leaves are shown (d). All data are presented as Mean $\pm$ SD. Different alphabets indicate statistically significant differences among different chlorotic leaves



547

548 **Fig. 5** In each panel upper side is a photograph of a leaf and lower side is an image of measured Fv/Fm
549 value. Adaxial side (**a–d**) and abaxial side (**e–h**) of green- (**a, e**), mottled- (**b, f**), chlorotic- (**c, g**) and
550 senescing-leaf (**d, h**) were shown. The scale bar is 5 cm. The color bar indicates Fv/Fm value from 0.65 to
551 0.85. In senescing leaves, Fv/Fm values of chlorotic section in both sides were too low and could not be
552 detected. In senescing leaf, there was no Fv/Fm value in the area which shows chlorosis on both sides of
553 the leaf. In such an area, color for the Fv/Fm value was shown by white. Photographs and two-dimension
554 images of Fv/Fm value data of abaxial side were flipped reversed up and bottom for easy comparison
555 with adaxial side

556

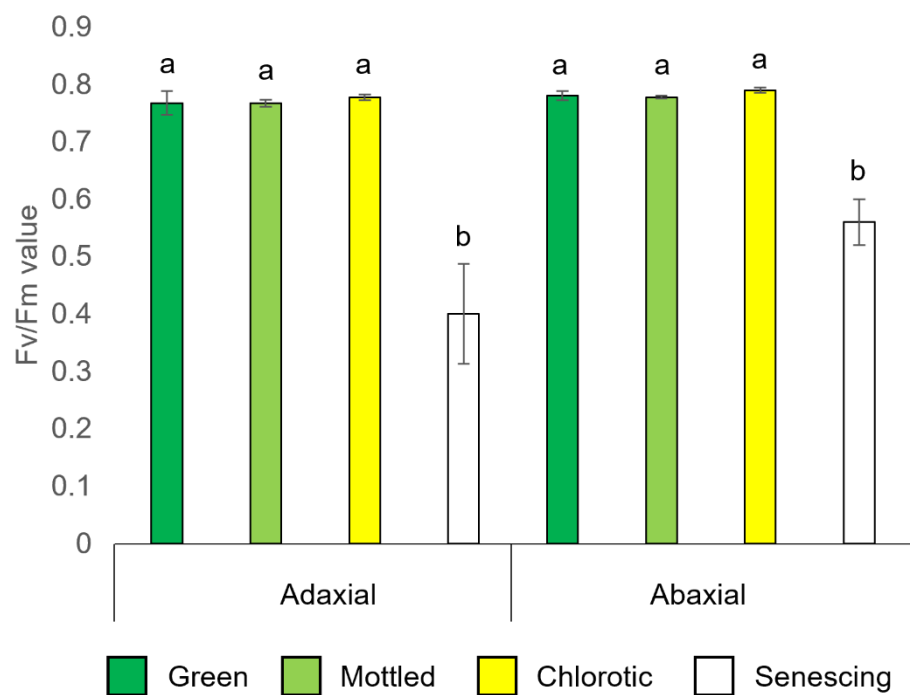


Fig. 6 Fv/Fm value of adaxial and abaxial side of the four types of leaves. Mean $\pm$ SD is shown. Different alphabets indicate the significant differences among different chlorotic leaves

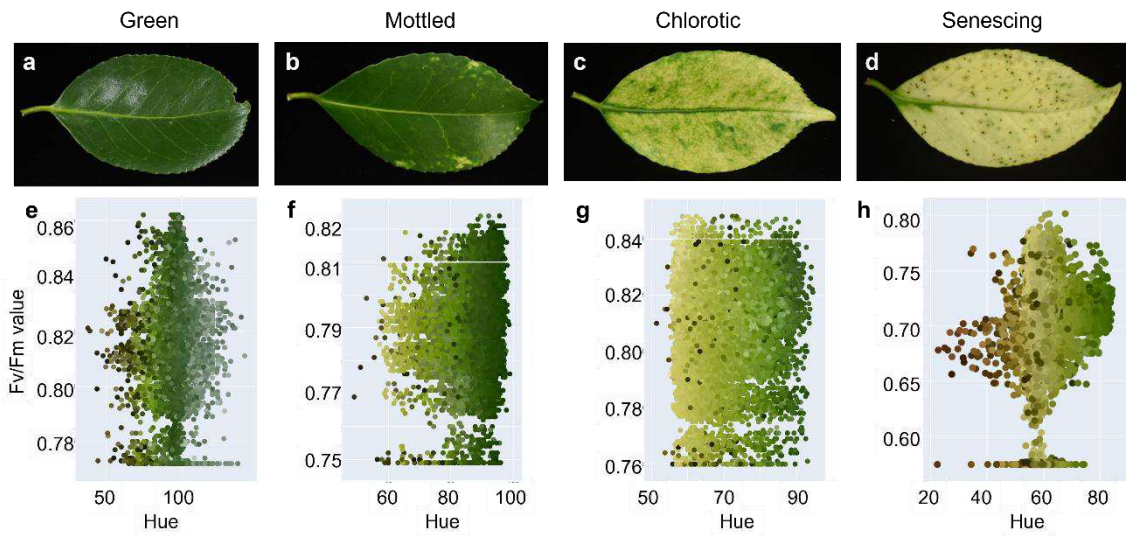


Fig. 7 A photograph of leaves (a–d). A scatter plot between hue value and Fv/Fm value of each pixel (e–h). The color of each plot indicates each pixel color. Regressions (e), $R^2 = 0.0857$; (f), $R^2 = 0.0281$; (g), $R^2 = 0.0136$; (h), $R^2 = 0.0307$

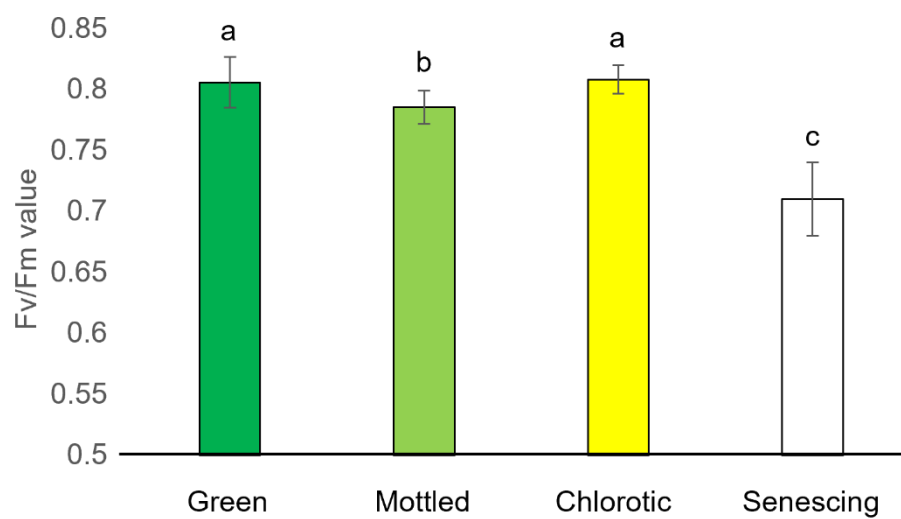


Fig. 8 Fv/Fm value of the inside of variegated section (hue value 60-65) in the four types of leaves. Mean $\pm$ SD is shown. Different alphabets indicate the significant difference among different chlorotic leaves

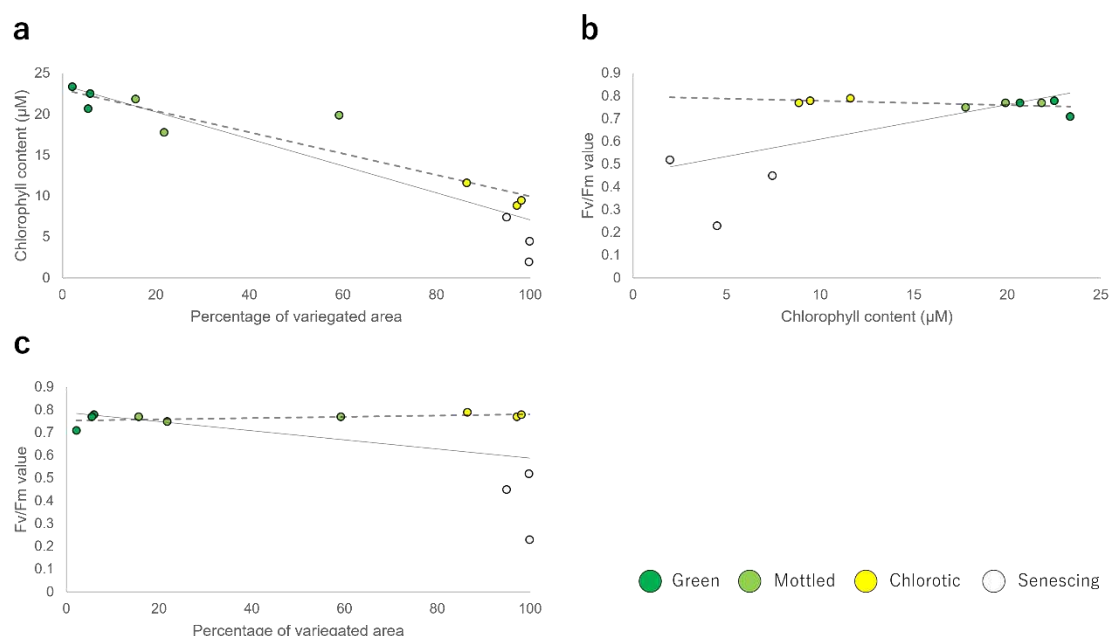
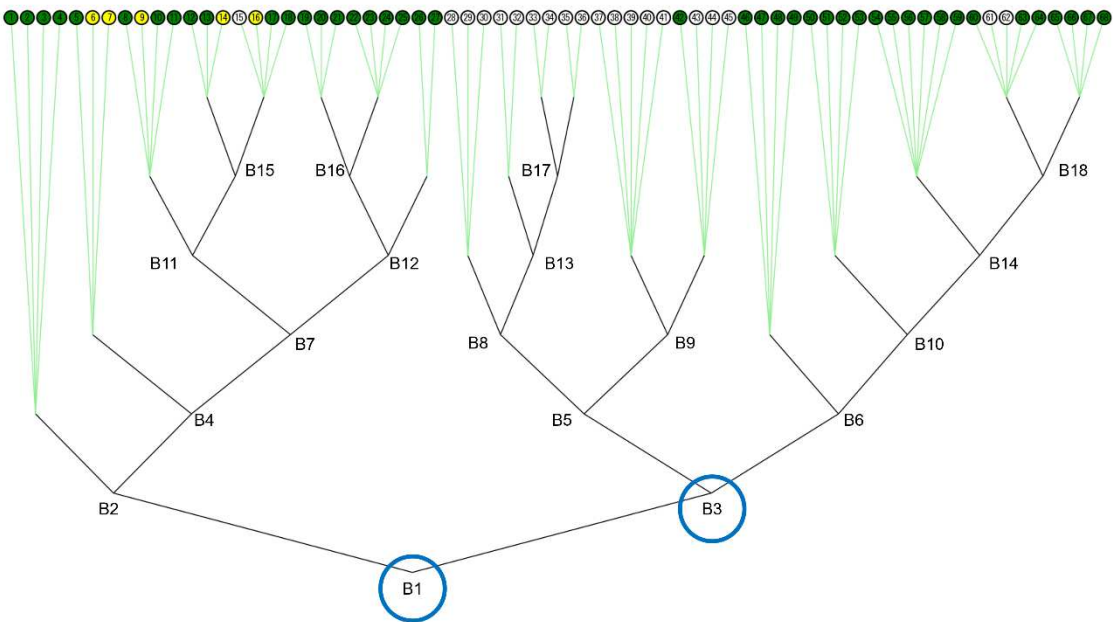


Fig. 9 Scatter plots between the percentage of variegated area and chlorophyll content (**a**), between chlorophyll content and Fv/Fm value (**b**), and between the percentage of variegated area and Fv/Fm value (**c**) were shown. The color of each plot indicates the type of leaf classified by the percentage of variegated area. Solid and dotted lines are regression lines of all leaf types and without senescing leaf type respectively.

Regressions (**a**), all: $Y = 23.559 - 0.1644X$, $R^2 = 0.8583$, $P\text{-value} < 0.001$, without senescing: $Y = 23.008 - 0.11304X$, $R^2 = 0.8546$, $P\text{-value} < 0.001$; (**b**), all: $0.4596 + 0.0152X$, $R^2 = 0.4226$, $P\text{-value} = 0.022$, without senescing: $Y = 0.7987 - 0.0019X$, $R^2 = 0.2208$, $P\text{-value} = 0.202$; (**c**), all: $Y = 0.7893 - 0.002X$, $R^2 = 0.2368$, $P\text{-value} = 0.109$, without senescing: $Y = 0.7531 + 0.0003X$, $R^2 = 0.2559$, $P\text{-value} = 0.165$

581



582

583 **Fig. 10** Circles and lines mean leaves and branches of 'Kumagai'. Green lines show petioles, and black
584 lines show branches. Colors of circles indicate a type of variegation; green, yellow and white indicate
585 green-, mottled- and chlorotic-leaves, respectively. The length of the lines does not indicate the distance
586 among leaves. They only show the branching patterns. Branching points with blue circle indicate points
587 where significant differences in variegation dispersion were observed (Online Resource 1). Each leaf was
588 numbered from 1 to 68 from left to right, and the percentage of variegated area is shown in Online
589 Resource 2
590

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ESM1.xlsx](#)
- [ESM2.xlsx](#)