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Positive correlation between tolerance to continuous UV-B irradiation, chloroplast numbers, chlorophyll content, and starch grain content in normal and variegated Japanese butterbur (*Petasites japonicus*) leaves

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ABSTRACT

A community containing both normal and variegated Japanese butterbur (*Petasites japonicus*) was found in a field at the Fukuoka University of Education campus in April 2012. Variegated Japanese butterbur leaves contained fewer chloroplasts, chlorophyll, and starch grains in green sectors, and lacked chloroplasts, chlorophyll, and starch grains in chlorotic (white) sectors, compared to those of normal Japanese butterbur. To investigate the effect of environmental factors on variegated Japanese butterbur leaves, both normal and variegated Japanese butterbur were irradiated with low (0.35 W m^{-2}) and high (1.67 W m^{-2}) levels of ultraviolet-B (UV-B). Our results indicate the following order of sensitivity to UV-B irradiation: white sectors in variegated Japanese butterbur leaves are the most sensitive to both low- and high-level UV-B irradiation, green sectors in variegated Japanese butterbur leaves are second, and green leaves of normal Japanese butterbur are third. Therefore, there is a positive correlation between tolerance to UV-B irradiation and content of chloroplasts, chlorophyll, and starch grains in Japanese butterbur leaves. Because starch grains are effective UV-B reflectors whereas chlorophylls are not efficient UV-B absorbers, starch might contribute primarily to UV-B tolerance. Solar UV-B irradiation might be one of the causes of scorching observed in white sectors of variegated Japanese butterbur leaves in the field.

Keywords: chlorosis, green sectors, white sectors.

I. INTRODUCTION

Ultraviolet-B (UV-B; 280–320 nm) irradiation has pleiotropic effects on development, morphology, and physiology of higher plants[1]. Exposure to UV-B leads to DNA and membrane damage, reduced photosynthetic activity, inhibition of hypocotyl elongation, stunted growth, reduced leaf area, bronzing, and necrosis[12), 13), 16)]; thus UV-B irradiation inhibits plant growth.

The variegated leaf phenotype characterized by distinct green and white sectors is commonly observed across a broad range of plant species[4), 6), 7)], and many of these variegated plants are highly valued as ornamental plants. The green sectors contain normal, photosynthetically active chloroplasts, whereas the white sectors contain plastids devoid of chlorophyll and/or carotenoids and photosynthetic reaction centers[4), 7)]. Hence, variegated leaves have reduced potential to fix carbon dioxide into sugars and, as a consequence, variegated plants tend to grow more slowly[3), 15)]. Potential differences in environmental adaptation between normal and variegated plants of the same species are not fully understood.

We identified a community containing both normal and variegated Japanese butterbur (*Petasites japonicus*) in a field at the Fukuoka University of Education (FUE) campus in April 2012. To investigate the effects of environmental factors on variegated Japanese butterbur leaves, both normal and variegated Japanese butterbur were replanted into clay pots and irradiated with low (0.35 W m^{-2}) and high (1.67 W m^{-2}) levels of UV-B. Differences of the effects of UV-B irradiation on normal and variegated Japanese butterbur leaves are discussed.

II. MATERIALS AND METHODS

1. Plant materials

Normal and variegated Japanese butterbur (*Petasites japonicus*) were used in this study. A community of normal Japanese butterbur (Fig. 1A) was found all over the field, whereas a community of variegated Japanese butterbur (Fig. 1B) was growing in a narrow area in front of one of the departmental buildings on the campus of FUE in April 2012. The origin of the variegated Japanese butterbur community is unknown. Both normal (n=10) and variegated (n=10) Japanese butterbur with rootstock were exhumed and transferred to clay pots (diameter = 20 cm) containing the soil composite Kumiai-Engei-Baido (0.4 g of N, 1.2 g of P, 0.2 g of K per kg; Seishin Sangyo Co. Ltd., Kitakyushu, Japan) (Fig. 1C). The plants were allowed to acclimatize for one week in the greenhouse with watering.

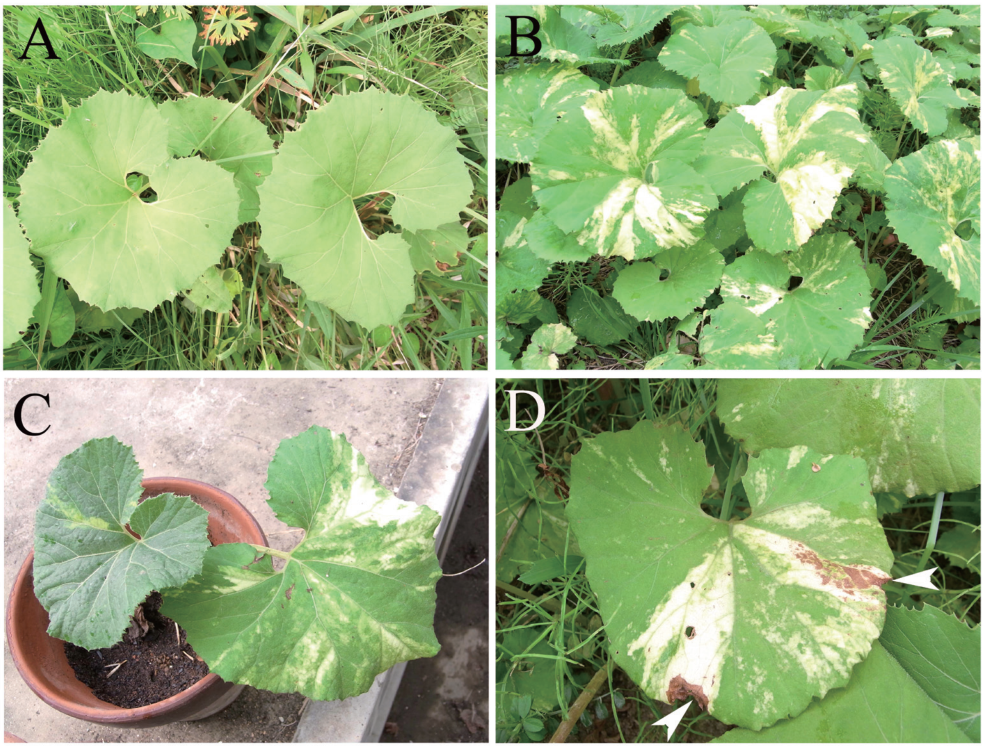

Fig. 1 Experimental plants in the field and after transferring to clay pots. (A) Normal Japanese butterbur in the field. (B) Variegated Japanese butterbur in the field. (C) Variegated Japanese butterbur transferred to clay pots (diameter = 20 cm). (D) Variegated Japanese butterbur in the field with scorched leaves. White arrowheads indicate scorching that progressed preferentially in white-leaf sectors. Plants were photographed in April 2012.

2. Observation of the leaves

To investigate the cytology of Japanese butterbur leaf variegation, leaves of both normal (n=3) and variegated (n=3) Japanese butterbur were cut into 1.0 cm square with a razor blade. For the variegated Japanese butterbur leaf, a sample containing both green and white sectors was prepared, and these samples were then observed under stereomicroscope (SPZT-50FTM; Carton Optical Industries, Ltd., Tokyo, Japan) and light microscope (ECLIPSE E600W; Nikon, Co., Tokyo, Japan).

To investigate starch grains, leaves of both normal (n=3) and variegated (n=3) Japanese butterbur were cut into 1.0 cm square with a razor blade. A sample containing both green and white sectors was prepared for the variegated Japanese butterbur leaf. The samples were treated with 10% (w/v) KOH until they were decolored[14]. After washing with distilled water, samples were stained with Lugol solution containing 0.67% (w/v) potassium iodide and 0.33% (w/v) iodine for 10 min at room temperature, and observed under stereomicroscope and light microscope as described above.

3. Determination of chlorophyll content

Chlorophyll *a*, *b*, and total chlorophyll contents in green leaves of normal Japanese butterbur, green sectors in variegated Japanese butterbur leaves, and white sectors in variegated Japanese butterbur leaves were measured according to a modified method of Porra et al. (1989)[5]. Briefly, 1.0 cm square of fresh leaves were eluted with 3 ml of 100% *N,N'*-Dimethylformamide (DMF) for 24 h at 4°C. Eluate was measured at 663.8 nm and 646.8 nm with a spectrophotometer (UVIDEC-4; JASCO Corporation, Tokyo, Japan) and the concentrations of chlorophyll *a*, *b*, and total chlorophyll were calculated as follows. Chlorophyll a (mg l^{-1}) $=12.00 O.D._{663.8} - 3.11 O.D._{646.8}$, Chlorophyll b (mg l^{-1}) $=20.78 O.D._{646.8} - 4.88 O.D._{663.8}$, Total chlorophyll (mg l^{-1}) $=17.67 O.D._{646.8} + 7.12 O.D._{663.8}$. Chlorophyll content was expressed as mg g^{-1} fresh weight (f.w.). This experiment was performed six times (three samples in each of two independent experiments), in green leaves of normal Japanese butterbur, green sectors in variegated Japanese butterbur leaves, and white sectors in variegated Japanese butterbur leaves, respectively.

4. UV-B irradiation

The acclimatized normal (n=10) and variegated (n=10) Japanese butterbur plants were transferred to a growth cabinet furnished with continuous fluorescent light (FLR40SW/M/36-B; Hitachi Ltd., Tokyo, Japan) at 25°C, with a photosynthetic photon flux density (PPFD) at the plant surface of approximately 160 μmol m^{-2} s^{-1}. For continuous low-level UV-B irradiation, a sunlamp (FL-20E; Tozai Densan Ltd., Osaka, Japan) was suspended 15 cm above the leaves of normal (n=5) and variegated (n=5) Japanese butterbur plants. Wavelengths below 290 nm were absorbed by covering the sunlamp with a polyvinyl chloride film (cutting sheet 000C; Nakagawa Chemical Inc., Tokyo, Japan). The films were replaced weekly due to decreases in transmittance. Low-level UV-B irradiation was conducted for 11 d. The UV intensity was measured using digital UV-intensity meters (UV-5.7, UV-6.2, and UV-8.0; MK Scientific Inc., Yokohama, Japan). The average intensity of low-level UV-B irradiation was 0.35 ± 0.06 W m^{-2} (Table 1).

Table 1 Intensity of UV irradiation that was delivered to Japanese butterbur leaves (measured 15 cm below the sunlamp).

	Intensity of UV irradiation		
	UV-C (W m^{-2}) (246–262 nm)	UV-B (W m^{-2}) (280–320 nm)	UV-A (W m^{-2}) (320–400 nm)
Sunlamp (high-level UV-B)	N.D.[a]	1.67 ± 0.08[b]	0.11 ± 0.03[b]
Sunlamp + polyvinyl chloride film (low-level UV-B)	N.D.[a]	0.35 ± 0.06[b]	0.10 ± 0.04[b]

[a] N.D., not detected.
[b] Values represent the means ± standard error at three different places for 7 d.

For continuous high-level UV-B irradiation, a sunlamp (FL-20E; Tozai Densan Ltd., Osaka, Japan) without polyvinyl chloride film was suspended 15 cm above the leaves of normal (n=5) and variegated (n=5) Japanese butterbur plants and the high-level UV-B irradiation was conducted for 5 d. UV intensity was measured using digital UV-intensity meters (UV-5.7, UV-6.2, and UV-8.0; MK Scientific Inc., Yokohama, Japan), and the average intensity of high-level UV-B irradiation was 1.67 ± 0.08 W m^{-2} (Table 1). In the present study, the effects of UV-A irradiation were not considered because the UV-A intensity was low (Table 1).

III. RESULTS AND DISCUSSION

A community containing both normal and variegated Japanese butterbur was found in a field at the FUE campus in April 2012 (Figs. 1A, 1B). The color of green sectors in variegated Japanese butterbur leaves cannot be visually distinguished from that of normal Japanese butterbur leaves (Figs. 1A, 1B). Variegated Japanese butterbur leaves have broad panels of chlorotic (white) sectors (Fig. 1B). We performed cytological and biochemical analysis of normal and variegated Japanese butterbur leaves (Figs. 2A, 2B, 2C, 2D, 3). Normal Japanese butterbur leaves contained numerous chloroplasts (Fig. 2E, black arrowheads) and starch grains (Fig. 2F, white arrowheads), whereas variegated Japanese butterbur leaves contained fewer chloroplasts (Fig. 2Ga, black arrowheads) and starch grains (Fig. 2Ha, black arrowheads) in green sectors, and no chloroplasts (Fig. 2Gb) or starch grains (Fig. 2Hb) in white sectors. The content of chlorophyll a, b, and total chlorophyll was 1.47, 0.48, and 1.95 mg g^{-1} f.w. in green leaves of normal Japanese butterbur, 0.68, 0.22, and 0.9 mg g-1 f.w. in green sectors of variegated Japanese butterbur leaves, 0.04, 0.01, and 0.05 mg g^{-1} f.w. in white sectors of variegated Japanese butterbur leaves (Fig. 3). Thus, variegated Japanese butterbur leaves contain fewer chloroplasts, chlorophyll, and starch grains in green sectors, and lack chloroplasts, chlorophyll, and starch grains in white sectors, compared to those of normal Japanese butterbur.

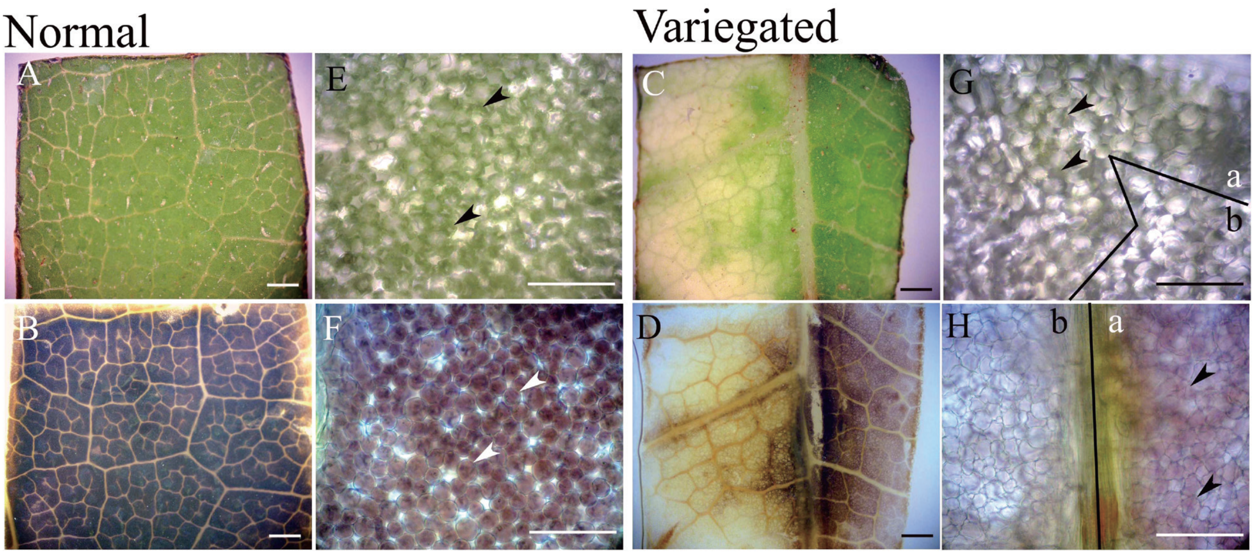

Fig. 2 Leaf surface in normal and variegated Japanese butterbur. (A) Leaf surface in normal Japanese butterbur. (B) Leaf surface in normal Japanese butterbur after decolorizing and staining with Lugol solution. (C) Leaf surface containing green and white sectors in variegated Japanese butterbur. (D) Leaf surface containing green and white sectors in variegated Japanese butterbur after decolorizing and staining with Lugol solution. (E) Magnified leaf surface in normal Japanese butterbur. Black arrowheads indicate chloroplasts. (F) Magnified leaf surface in normal Japanese butterbur after decolorizing and staining with Lugol solution. White arrowheads indicate starch grains. (G) Magnified leaf surface containing green (a) and white (b) sectors in variegated Japanese butterbur. Black arrowheads indicate chloroplasts. (H) Magnified leaf surface containing green (a) and white (b) sectors in variegated Japanese butterbur after decolorizing and staining with Lugol solution. Black arrowheads indicate starch grains. Scale bars in (A), (B), (C), and (D) is 1.0 mm. Scale bars in (E), (F), (G), and (H) is 0.1 mm.

To investigate the effect of environmental factors on Japanese butterbur leaves, both normal and variegated Japanese butterbur exhumed from the field-grown community were replanted into clay pots, allowed to acclimatize, and then irradiated with low-level (0.35 W m^{-2}) UV-B (Table 1). No visible changes were found in normal Japanese butterbur leaves after continuous low-level UV-B irradiation for 1–11 d (Fig. 4). By contrast, scorch progressed preferentially in white sectors in variegated Japanese butterbur leaves after continuous low-level UV-B irradiation for 3–11 d (Fig. 4, white arrowheads). The adjacent green sectors became white after continuous low-level UV-B irradiation for 7-9 d (Fig. 4, black arrowheads), and became scorched by 11 d (Fig. 4). These results suggest that continuous low-level UV-B irradiation of variegated Japanese butterbur leaves induces chlorosis in green-leaf sectors, and leads to the propagation of scorching, which originated in white-leaf sectors, into the newly chlorotic sectors. Thus, UV-B-induced white (chlorotic) sectors should be distinguished from inherent white sectors in variegated Japanese butterbur leaves. It remains a possibility that continuous low-level UV-B irradiation for more than 11 d could cause chlorosis, leading to scorching in normal Japanese butterbur leaves. In summary, the results indicate the following order of sensitivity to low-level UV-B irradiation: white sectors in variegated Japanese butterbur leaves are the most sensitive to low-level UV-B irradiation, green sectors in variegated Japanese butterbur leaves are second, and green leaves of normal Japanese butterbur are third. In normal Japanese butterbur, the surface of developing leaves (Fig. 5A) became rough compared to that of unfolded leaves (Fig. 5B) after continuous low-level UV-B irradiation for 1–11 d. This tendency was not observed on the surface of developing leaves of normal Japanese butterbur grown under natural field conditions (data not shown). These results indicate that developing leaves are more sensitive to low-level UV-B irradiation compared to unfolded leaves in normal Japanese butterbur.

To investigate the effects of different intensity of UV-B irradiation on the leaves of both normal and variegated Japanese butterbur, we next treated normal and variegated Japanese butterbur leaves with high-level (1.67 W m^{-2}) UV-B irradiation (Table 1). The leaf surface of normal Japanese butterbur

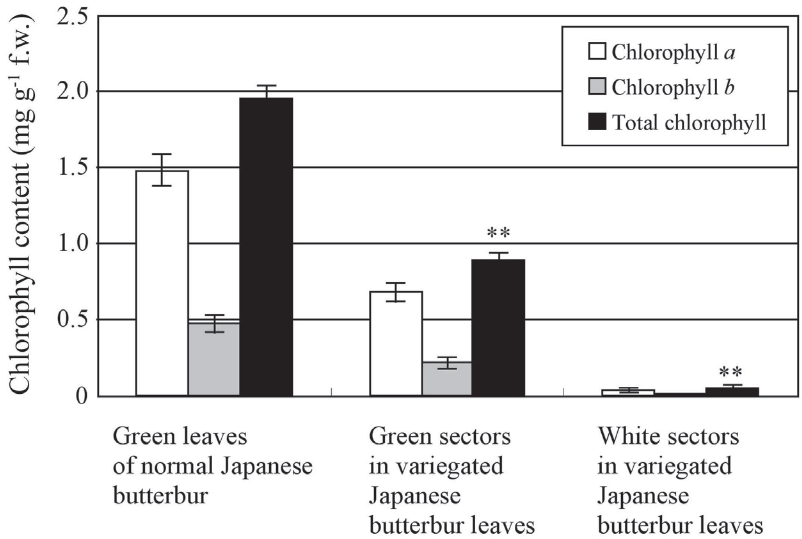

Fig. 3 Chlorophyll *a*, *b*, and total chlorophyll contents in green leaves of normal Japanese butterbur, green sectors in variegated Japanese butterbur leaves, and white sectors in variegated Japanese butterbur leaves. The values are expressed as the average of six replicates (three samples in each of two independent experiments) and bars indicate ± standard error. Double asterisk in the green sectors in variegated Japanese butterbur leaves indicates a significant difference between green leaves of normal Japanese butterbur and green sectors in variegated Japanese butterbur leaves ($P < 0.01$, Student's *t*-test). Double asterisk in the white sectors in variegated Japanese butterbur leaves indicates a significant difference between green sectors in variegated Japanese butterbur leaves and white sectors in variegated Japanese butterbur leaves ($P < 0.01$, Student's *t*-test). Chlorophyll content was expressed as mg g^{-1} fresh weight (f.w.).

became dark brown after continuous high-level UV-B irradiation for 3-5 d (Fig. 6). By contrast, scorch progressed preferentially in white sectors in variegated Japanese butterbur leaves after continuous high-level UV-B irradiation for 1-5 d (Fig. 6, white arrowheads). The adjacent green sectors became dark brown after continuous high-level UV-B irradiation for 2-5 d (Fig. 6), which appeared to originate from the white sector; thus continuous high-level UV-B irradiation did not induce chlorosis in the green sectors (Fig. 6, white arrowheads). The extent of damage from continuous high-level UV-B irradiation is higher in variegated Japanese butterbur leaves compared to that in normal Japanese butterbur. It appears that the dark brown parts in leaves are caused by rapid scorching in response to continuous high-level UV-B irradiation. These results indicate that white sectors in variegated Japanese butterbur leaves have the highest sensitivity to high-level UV-B irradiation, followed by green sectors in variegated Japanese butterbur leaves and green leaves of normal Japanese butterbur.

UV-B irradiation with either low or high levels produces consistent results with respect to the

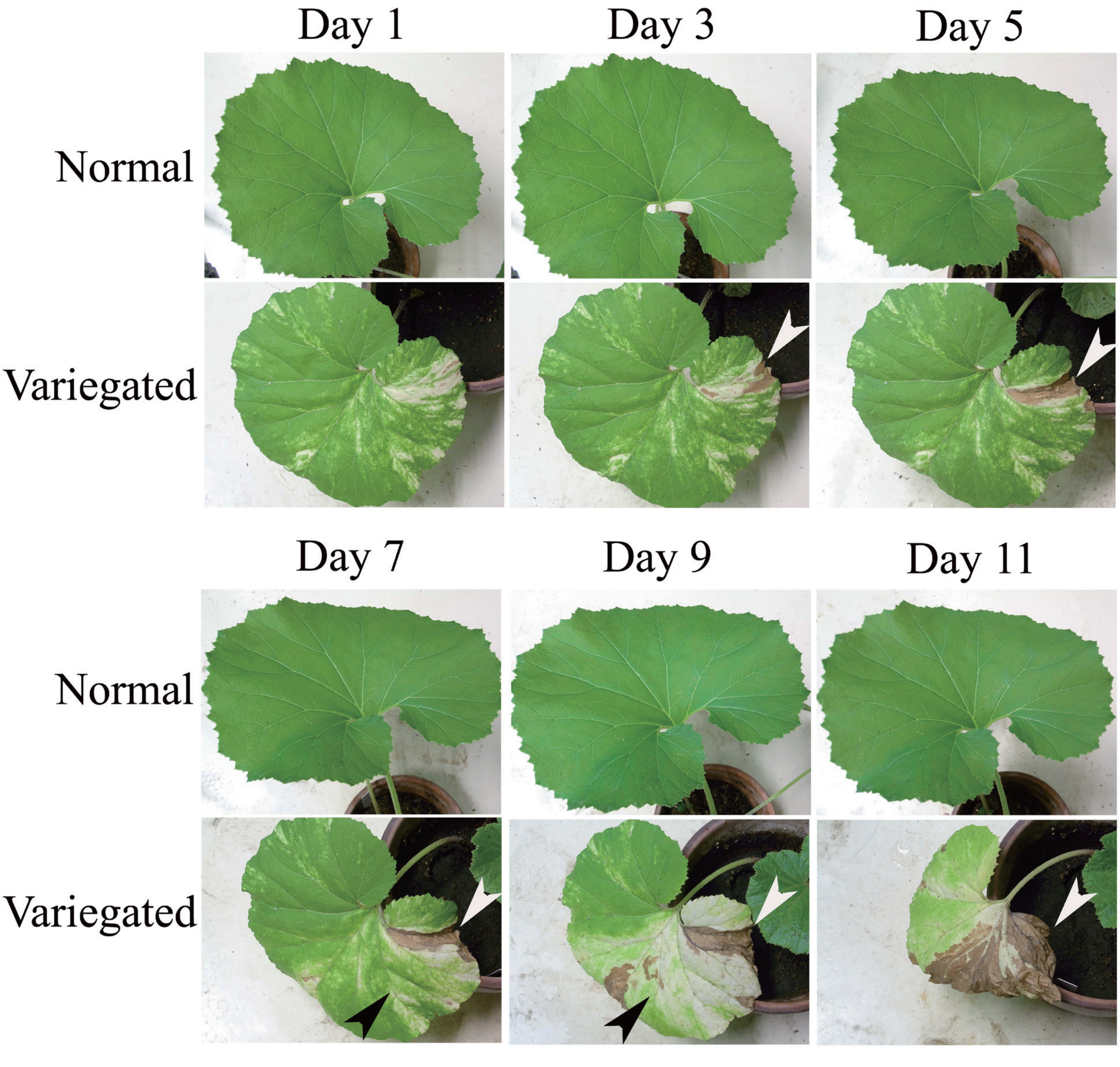

Fig. 4 Effect of continuous low-level (0.35 W m^{-2}) UV-B irradiation for 11 d on normal and variegated Japanese butterbur leaves. White arrowheads indicate scorch, which progressed preferentially at white sectors in variegated Japanese butterbur leaves. Black arrowheads indicate white (chlorotic) sectors that were caused by continuous low-level UV-B irradiation in green sectors of variegated Japanese butterbur leaves.

sensitivity of Japanese butterbur leaves. Therefore, there is a positive correlation between sensitivity to UV-B irradiation, chloroplast numbers, chlorophyll content, and starch grain content in the leaves of both normal and variegated Japanese butterbur (Figs. 2, 3, 4, 6). Chlorophyll is a major phtosynthesis pigment. Its major function in photosynthesis is related to light collection and light conversion processes[10]. Chlorophylls are not efficient UV-absorbers but are able to absorb UV irradiation, especially around 350 nm, UV-A[17]. It is reported that chlorophyll content did not correlate with sensitivity to UV-B irradiation in a variety of vegetable plants[11]. In the adaxial surface of the yellow petals of *Ranunculus japonicus*, first layer contains carotenoid and chlorophylls and second layer contains starch grains[2]. Hariyama et al. (2013)[2] cleared that starch grains in the second layer reflect UV (280-400 nm; UV-A and UV-B) irradiation. Thus, starch grains are effective UV-B reflectors whereas chlorophylls are not efficient UV-B absorbers. Similarly, starch grains might contribute primarily to UV-B tolerance in the leaves of both normal and variegated Japanese butterbur. Because starch is produced by photosynthesis in chloroplasts, chloroplasts and chlorophylls might contribute secondarily to UV-B tolerance.

In many variegated Japanese butterbur growing under natural field conditions, scorch progresses preferentially in white sectors of leaves (Fig. 1D, white arrowheads), whereas no scorching was observed in young leaves of normal Japanese butterbur growing under natural field conditions. These observations suggest that white sectors in variegated Japanese butterbur leaves are more sensitive to adverse environmental factors compared to the green sectors in normal or variegated Japanese butterbur leaves. This estimation strongly supports, and is consistent with, previous reports that variegated mutants are highly vulnerable to photodamage under high light[8), 9)]. The observed scorch morphology in variegated Japanese butterbur leaves is similar to the low-level UV-B-induced leaf damage we observed in the potted experimental plants (Fig. 4, white arrowheads). Therefore, solar UV-B irradiation might be one

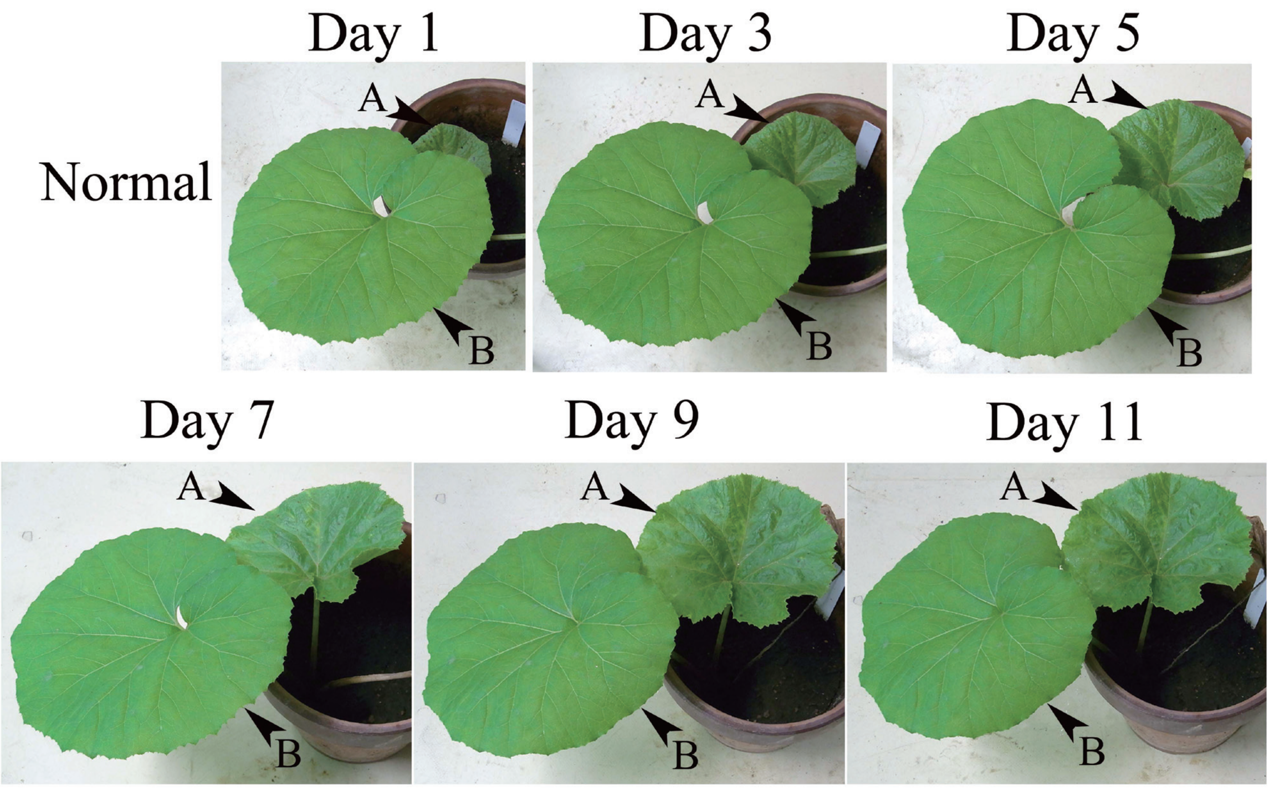

Fig. 5 Effect of continuous low-level (0.35 W m^{-2}) UV-B irradiation for 11 d on developing (A) and unfolded (B) leaves of normal Japanese butterbur.

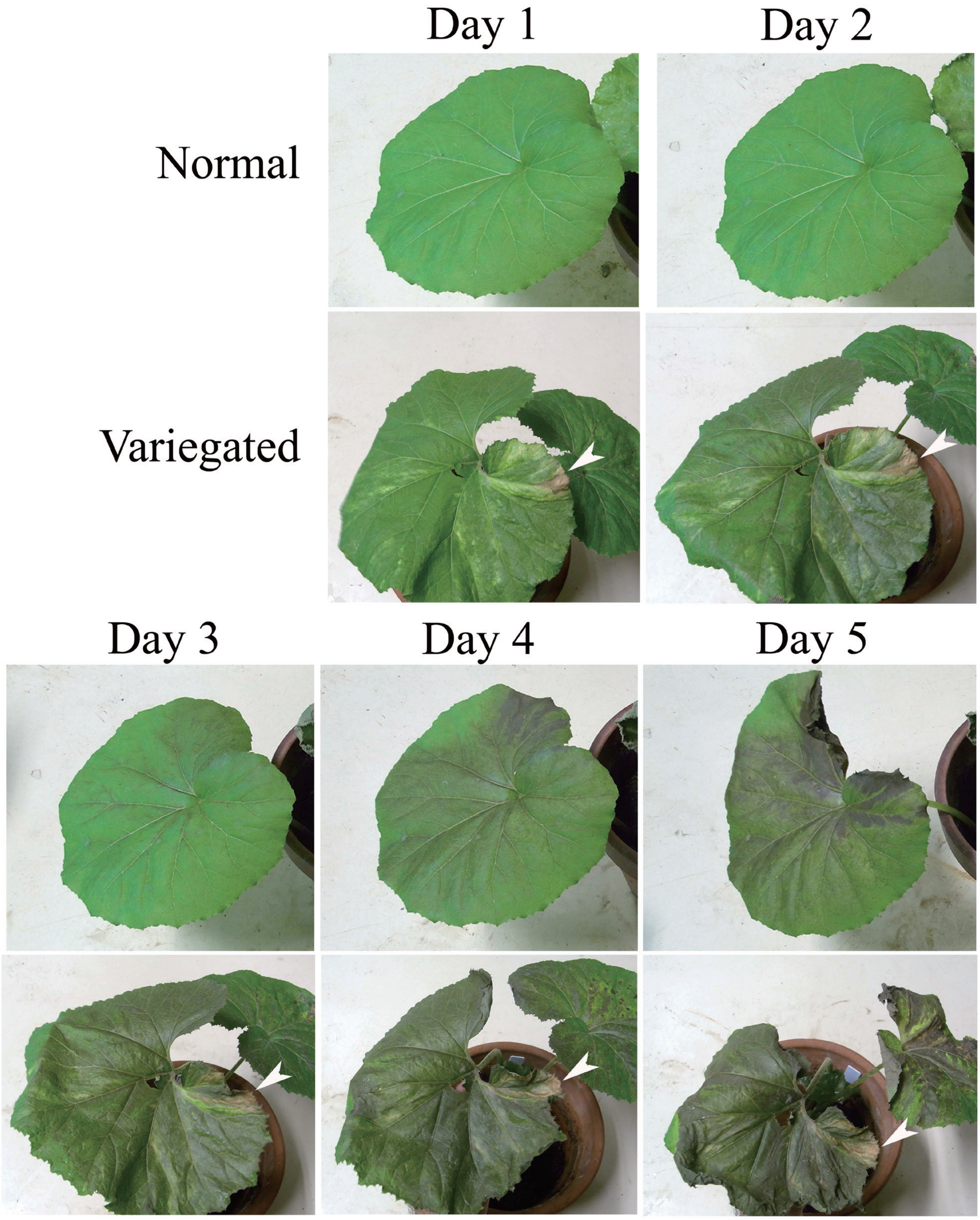

Fig. 6 Effect of continuous high-level (1.67 W m^{-2}) UV-B irradiation for 5 d on normal and variegated Japanese butterbur leaves. White arrowheads indicate scorch that progressed preferentially at white sectors in variegated Japanese butterbur leaves.

of the causes of scorch in white sectors of variegated Japanese butterbur leaves growing under natural field conditions. However, because the surface of developing leaves of normal Japanese butterbur grown under natural field conditions did not become rough like that of normal Japanese butterbur grown under continuous low-level UV-B irradiation (Fig. 5), the effect of UV-B irradiation under natural field conditions might be lower compared to that under continuous low-level UV-B irradiation in a growth cabinet. The growth differences between normal and variegated Japanese butterbur in the field were not observed (Figs. 1A, 1B) although variegated plants are reported to grow more slowly than normal plants[3), 15)]. The mechanism that enables variegated Japanese butterbur to grow as well as normal Japanese butterbur under field conditions, in spite of the reduced content of chloroplasts, chlorophyll, and starch grains, should be determined in future research.

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