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UV-B protective effect of a polyacylated anthocyanin, HBA, in flower petals of the blue morning glory, *Ipomoea tricolor* cv. Heavenly Blue

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Abstract—The protective effects of polyacylated anthocyanin, heavenly blue anthocyanin (HBA), in blue flower petals of morning glory (*Ipomoea tricolor* cv. Heavenly Blue) against UV-B induced DNA damage were examined. We first clarified the concentration of HBA in epidermal vacuoles to be 12 mM, and then constructed a UV-B irradiating apparatus resembling flower petal tissue to assess the screening effect of HBA. Monochromatic (280 and 310 nm) or broad UV-B induced DNA lesions were reduced completely by the HBA filter to the same molecular numbers as those in living petal epidermis. However, diluted HBA solution and trisdeacyl HBA did not have the same reduction effect. HBA was more tolerant to solar radiation than trisdeacyl HBA. These data strongly suggest that polyacylated anthocyanins in flower petals can screen harmful UV-B efficiently. This action might be largely due to aromatic acyl residues.

1. Introduction

Sunlight is essential for plants at all life stages, but solar radiation also contains harmful UV-B (280–320 nm). Although most UV-B radiation is absorbed by the ozone layer, the recent depletion of the latter has generated a serious problem for all living organisms on the Earth.[1–3] Phenolic second metabolites such as flavonoids and cinnamic acid derivatives are accumulated in epidermal cells by UV irradiation.[3–7] Therefore, those compounds have been considered as effective shielding materials.[3–10] However, direct evidence of the screening effects of flavonoids against UV-B induced DNA damage has only been presented by Kootstra, using solutions of rutin, naringenin, and apple extracts.[10]

Biosynthesis of anthocyanin, a colored polyphenol compound, which is responsible for the red, through purple to blue colors of flowers, leaves, and fruits, is also induced by UV radiation.[11–13] UV irradiation of leaves, fruits, and stems usually causes red- to purple-colored tissues. Actually, red color of apple skin arises from radiation. These are due to the accumulation of anthocyanins, suggesting that they play some protective role.[12–14] Furthermore, aromatic acyl residues of polyacylated anthocyanins absorb UV-B light overlapping the absorption spectrum of DNA; therefore, those pigments are expected to be an effective UV-screening pigment in plants.[14]

Recently we reported that polyacylated anthocyanins are more tolerant to UV-B than nonacylated anthocyanins.[15–17] Especially, heavenly blue anthocyanin (HBA, **1**, Fig. 1)[18] isolated from blue petals of the morning

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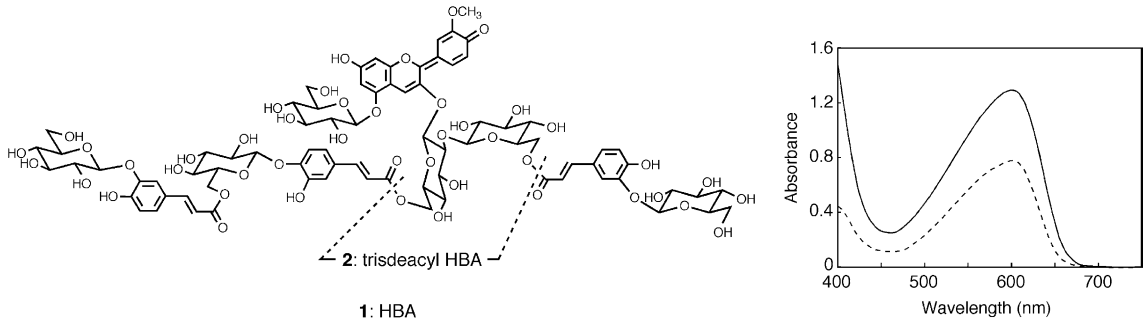

Figure 1. (a) Left: structures of heavenly blue anthocyanin (HBA, **1**) and trisdeacyl HBA (**2**). (b) Right: reflection spectrum of blue open flower petals recorded with an integral sphere apparatus and the absorption spectrum of blue protoplasts prepared from blue petals. (—): flower petal, (---): protoplast.

glory, *Ipomoea tricolor* cv. Heavenly Blue, showed interesting stability under strong UV irradiation. HBA was more stable in physiological pH (pH 7.5) than strong acidic or weakly acidic solution, even though anthocyanin is usually stable in strong acidic media but very unstable in alkaline media.[16] These results suggest that petal anthocyanins must play some biological role in protecting petal tissues from solar radiation. Thus, we studied the screening effects of HBA against UV-B induced damage. To assess the role of petal pigment, we first measured the concentration of **1** in epidermal vacuoles, and then constructed an irradiating system modeling a petal tissue. Using the system, a DNA solution was irradiated with UV-B through an HBA filter, subsequently the DNA damage was quantified using enzyme-linked immunosorbent assay (ELISA). In this report, we present the protective effect of **1** and also discuss the effects of concentration and caffeoyl residues on the UV-B screening role.

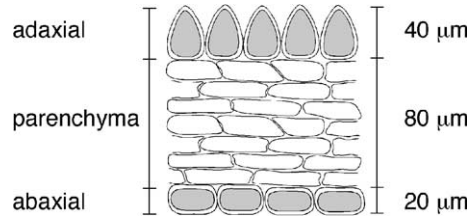

Figure 2. Schematic model of petal tissue of blue morning glory.

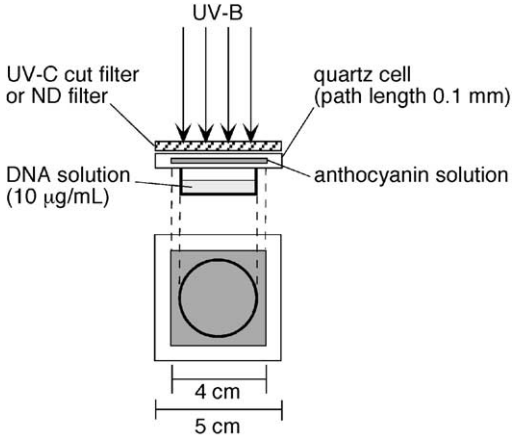

Figure 3. The system for irradiation of UV-B onto DNA solution through an anthocyanin screen.

2. Results and discussion

2.1. Concentration of HBA in colored epidermal cells of blue morning glory

In flower petals, anthocyanins are generally localized in the central vacuoles of cells of both epidermal layers.[19] In the blue morning glory petals, the colored cells are also localized at both epidermal layers.[20] Red buds and blue open petals contain the same anthocyanin, **1** (Fig. 1a)[18], and the difference in petal color is caused by an unusual vacuolar pH increase from 6.6 to 7.7.[20] The adaxial epidermal cells of petals are conical and the abaxial epidermal cells are flat (Fig. 2). Therefore, direct measurement of vacuolar HBA concentration is very difficult. By enzymatic treatment with cellulase and pectinase, we prepared blue colored protoplasts from petals without any color change, then the absorption spectrum of a single cell in an isotonic solution was measured by micro-spectrophotometry with a 10 μm diameter optical light flux.[19] The diameter of colored cells was 20–30 μm and their λ_{vismax} was 599 nm, identical to that for blue open flower petals (Fig. 1b). Since colored vacuoles occupy more than 95% of the cell volume, the diameter of the vacuole is almost equal to that of the cell. HBA is a unique pigment in the petals and the molar absorption coefficient of **1** is not affected by the concentration because **1** has no intermolecular interaction.[21] Therefore, the concentration in the living protoplast was calculated directly by a calibration curve of standard solutions. Ten protoplasts were selected by random sampling and the average concentration of **1** in blue protoplasts was determined to be 12 mM. The λ_{vismax} for red protoplasts prepared from buds (diameter: ca. 20 μm) was 559 nm and the average concentration of **1** was ca. 23 mM. The decrease in concentration of **1** during blooming might be caused by the twofold enlargement of vacuolar volume by water flux. Before our study, only one research was reported on the measurement of anthocyanin concentration in flower petal vacuoles. In the report Asen et al. presumed the concentration to be approximately 10 mM by

measuring the absorbance of the peeled epidermis of Iris petals.[22] Here, we determined accurate concentrations of HBA in epidermal vacuoles of open and bud petals.

2.2. UV irradiation apparatus

To assess the protective effects of petal anthocyanin against UV-B induced DNA damage, we constructed an experimental apparatus resembling petal tissue (Fig. 3). In 1994, Kootstra used a similar idea to examine the effects of flavonol and plant extracts against UV-B induced damage. However, the path length of the sample solution was ambient, therefore, the effects of flavonoids on the prevention of DNA damage were not quantitatively discussed in detail.[10] In our apparatus, a thin quartz cell resembles the epidermal layer, therefore, quantitative study can be conducted. Because of practical problems, we could not provide a quartz cell with a path length of 40 μm or less, therefore, the path length was fixed at 100 μm, then the diluted HBA (5 mM) was poured into the cell to equalize the absorbance. A DNA solution in a plastic petri dish (35 mm ϕ) was irradiated with monochromatic UV-B generated by the Okazaki large spectrograph (OLS)[23] or broad UV-B light by FL20SE from the top. UV light shorter than 290 nm was cut off using a UV-29 filter and the radiation dose was controlled by an ND filter.

2.3. Protection of UV-B induced DNA damage by HBA

The protective effect of purified **1** against UV-B was analyzed as the amount of DNA lesions, cyclobutane pyrimidine dimers (CPDs), and (6-4)photoproducts (6-4PPs), quantified by ELISA using specific monoclonal antibodies.[24] Since anthocyanin is a pH sensitive pigment,[20,21,25] the visible absorption spectra of **1** differ depending to the pH (Fig. 4). To differentiate the effect of the visible and UV absorption band, we studied the UV-B protective effect of various solutions. The screening effect of **1** against monochromatic UV-B at 280 or 310 nm generated by the OLS is shown in Figure 5 and Table 1. By irradiating at 280 nm without an HBA filter, the amounts of DNA lesions increased linearly to 60 J/m^2 depending on the UV dose. However, DNA damage was remarkably reduced with **1** (5 mM) in any solution (Fig. 5).

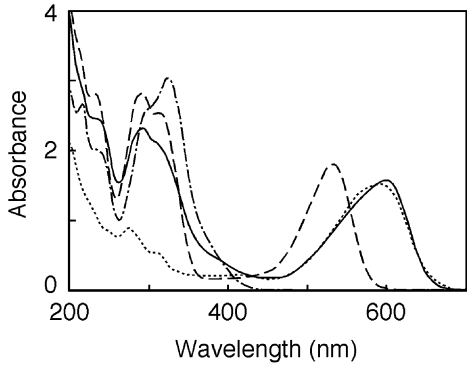

Figure 4. UV/vis spectra of HBA (**1**), trisdeacyl HBA (**2**), and methyl caffeate (**3**) in various solutions (path length: 0.1 mm). (—): **1** (5 mM, pH 7.5), (---): **1** (5 mM, TFA–methanol), (···): **2** (5 mM, pH 7.5), (-·-·): **3** (15 mM, pH 7.5).

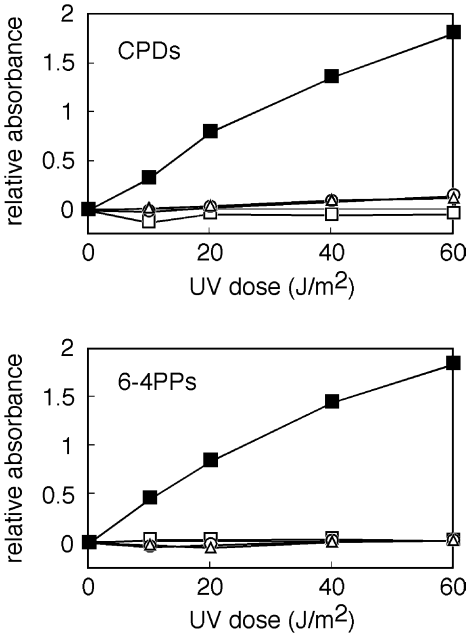

Figure 5. Prevention of DNA lesions induced by monochromatic UV-B (280 nm) with an HBA screen (5 mM). Data represent the average ± standard deviation of three experiments. (■): Control, (□): pH 7.5, (○): 0.5% TFA–H_2O, (△): 0.5% TFA–MeOH. Upper: CPDs; lower: 6-4PPs.

Table 1. Reduction of monochromatic UV-B induced photoproducts by HBA (5 mM) in various solutions

	Relative amounts (%)			
	280 nm[a]		310 nm[b]	
	CPDs	6-4PPs	CPDs	6-4PPs
pH 7.5	0 ± 2	1 ± 1	0 ± 1	3 ± 2
TFA–H_2O	9 ± 1	1 ± 1	13 ± 2	2 ± 1
TFA–MeOH	10 ± 4	1 ± 1	10 ± 9	2 ± 1

The data is represented as a relative amount of each photoproduct compared to the control experiment.
[a] 60 J/m^2.
[b] 20 kJ/m^2.

Similar results were obtained when DNA solutions were irradiated with UV-B at 310 nm (20 kJ/m^2). All the HBA solutions (5 mM) suppressed the DNA damage, too (Table 1). The transmittance of UV-B at 280 or 310 nm through the solution may be less than 1%; therefore, the actual radiation energy might be less than 0.6 J/m^2 at 280 nm and 0.2 kJ/m^2 at 310 nm, respectively. Actually, the amounts of DNA damage could be almost zero.

Because the radiation spectrum of the FL20SE lamp is broad and more similar to solar radiation than monochromatic UV-B, FL20SE is suitable for assessing the screening effects of anthocyanins in nature. Figure 6 shows the protective effect of HBA solution against UV-B (450 J/m^2). The filter of **1** (5 mM) completely

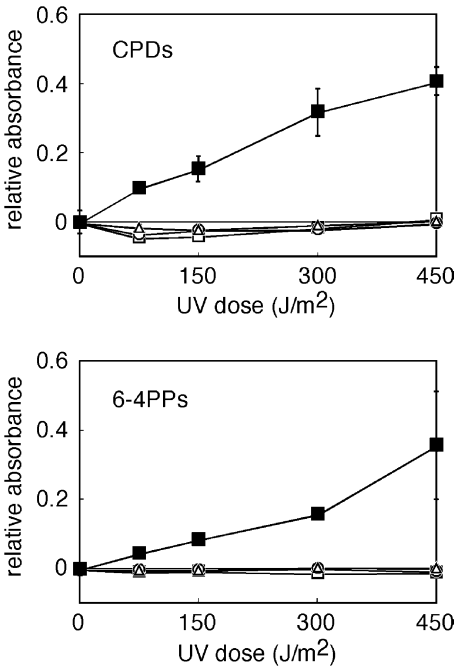

Figure 6. Prevention of DNA lesions induced by broad UV-B with an HBA screen (5 mM). Data represent the average ± standard deviation of three experiments. (■): Control, (□): pH 7.5, (○): 0.5% TFA–H_2O, (△): 0.5% TFA–MeOH. Upper: CPDs; lower: 6-4PPs.

Table 2. Reduction of broad UV-B[a] induced photoproducts by various concentrations of HBA in aqueous solution at pH 7.5

HBA (mM)	Relative amount (%)	
	CPDs	6-4PPs
5.0	5 ± 2	0 ± 7
0.5	53 ± 13	51 ± 1
0.05	86 ± 11	69 ± 4

The data is represented as a relative amount of each photoproduct compared to the control experiment.
[a] 450 J/m^2 by FL20SE.

suppressed the induction of CPDs and 6-4PPs similar to the results of monochromatic UV-B. In Table 2 the effect of diluted HBA solutions is shown. The protective effect of diluted HBA screen (0.5 and 0.05 mM at pH 7.5) decreased to one half or less indicating that the intensity of the absorption band at the UV-B region was crucial.

To clarify the contribution of intramolecular caffeoyl residues, the protective effects of trisdeacyl HBA (**2**) were studied (Table 3). The amount of DNA damage filtered with **2** (5 mM) was 20–40% higher than that of **1** (5 mM), but the addition of 3 molar equiv of methyl caffeate (**3**) recovered the screening effects (Table 3). The protecting effect of solo **3** (5 mM) in aqueous solution at pH 7.5 did not showed enough protecting effect. But 15 mM of **3** could prevent UV-B induced DNA damage nearly the same level as those of **1** and the mixture of **2** and **3** (3 equiv). Therefore, the screening effect depends mainly on UV-B absorbing residues. The difference of the protecting effect may be caused by the difference of stability of UV-absorbing compounds.

Table 3. Reduction of broad UV-B[a] induced photoproducts by trisdeacyl HBA (**2**) and methyl caffeate (**3**)

Compound[b]	Solvent	Relative amounts (%)	
		CPDs	6-4PPs
2	pH 7.5	35 ± 2	45 ± 12
2	TFA–H_2O	40 ± 6	29 ± 11
2	TFA–MeOH	32 ± 8	32 ± 11
2 + **3** (15 mM)	pH 7.5	0 ± 1	0 ± 3
3 (5 mM)	pH 7.5	31 ± 1	22 ± 3
3 (15 mM)	pH 7.5	10 ± 1	0 ± 7

The data is represented as a relative amount of each photoproduct compared to the control experiment.
[a] 450 J/m^2 by FL20SE.
[b] In all the experiments concentration of **2** was adjusted to be 5 mM.

Compound **3** coexisting with **2** may be stabilized by a co-pigment effect.

2.4. Stability of HBA under solar radiation

Because UV-B absorbing compounds must be more or less degraded for a long irradiation, the stability against UV radiation is important to show the continuous screening effect. We previously reported that diluted HBA solution in physiological pH (0.05 mM, pH 7.5) was more tolerant than that in acidic methanol under UV radiation of a high-pressure mercury arc.[16] This phenomenon was caused by the quenching of radiation as a fluorescence, which arises from the energy transfer from aromatic acyl residues to the anthocyanidin nucleus based on intramolecular stacking.[16] With this mechanism, polyacylated anthocyanins should express more effective UV protection than simple anthocyanins. To compare the stability of HBA (**1**) and trisdeacyl HBA (**2**) in the same condition as in petals, each solution (5 mM at pH 7.5) was exposed to solar radiation, and then the stability was analyzed by HPLC (Fig. 7). HBA was more stable than **2** indicating that polyacylated anthocyanin might be stabilized by intramolecular stacking. HBA was degraded only 20% by a solar UV-B dose of 6 kJ/m^2, corresponding to 6 h of outside exposure in November. Methyl caffeate (**3**) was more labile than anthocyanins (**1**, **2**) by UV light, therefore, the intramolecular acyl residues in petal anthocyanins should posses some function for UV-B screen.

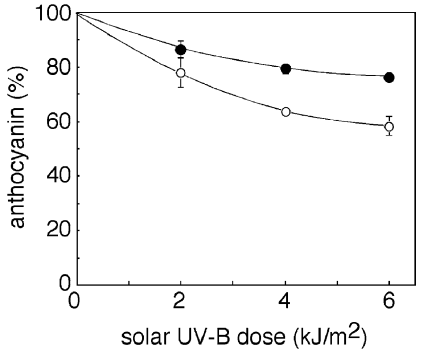

Figure 7. Stability of HBA (**1**) and trisdeacyl HBA (**2**) (5 mM, pH 7.5) under solar radiation. Data represent the average ± standard deviation of three experiments. (●): **1**, (○): **2**.

3. Conclusion

In conclusion, we first determined the concentration of HBA (**1**) in living petal cells, then, using apparatus resembling petal tissue, the UV protective effects of **1** were studied. The concentrated solution of **1** (5 mM) with the same mole number as in petals prevented DNA damage completely, though the effect of diluted **1** (0.5 mM and less) and trisdeacyl HBA (**2**) was low. Methyl caffeate (**3**, 15 mM) showed the UV-B screening effect indicating that acyl residue is essential for protection. Being taken account of the stability against UV-B polyacylated anthocyanins have more efficient protective effect than nonacylated pigment and UV-B absorbing compounds. The 20 kJ/m^2 irradiation of monochromatic UV-B radiation (310 nm) corresponds to one half the solar UV-B dose per day in central Japan[26] and 450 J/m^2 irradiation by FL20SE might be equivalent to a half hour exposure at noon in July.[27] Considering the irradiation dose and the stability of **1** under sun light, the screening effect of **1** in the epidermal cells may be sufficient to assume the UV-B protective function in vivo.

4. Experimental

4.1. Materials

HBA (**1**, TFA salts, 555 mg) was purified from frozen petals of the morning glory, *Ipomoea tricolor* cv. Heavenly Blue (5.1 kg, cultivated at the experimental farm of Nagoya University) according to the modified method described by Kondo et al.[18] The purity of HBA was determined by HPLC to be >98%. λ_{vismax} (pH 7.5): 599 nm (ε = 20,000), λ_{uvmax} (pH 7.5): 355 nm (ε = 40,000). Trisdeacyl HBA (**2**, TFA salts, 94 mg) was obtained by alkaline hydrolysis of **1** (560 mg).[28] Caffeic acid (102 mg, Sigma Chemical Company, St. Louis, MO) was esterified with acidic methanol to give the methyl ester (**3**, 109 mg). Calf thymus DNA was purchased from the Sigma Chemical Company.

4.2. Concentration of HBA in a living vacuole

Fresh petals of the blue open flowers and purple buds of morning glory, *Ipomoea tricolor* cv. Heavenly Blue, were treated with a solution of 1.0% (w/v) Sumizyme C (Shin-Nihon Kagaku Industries, Anjyo, Japan), 0.1% (w/v) Pectolyase Y-23 (Kikkoman, Noda, Japan) and mannitol (0.60 M, at pH 7.0 for open flowers, 0.65 M, at pH 6.5 for buds) to give colored protoplasts according to the methods of Yoshida et al.[19a] with slight modification. Concentrations of HBA in vacuoles were estimated by microspectrophotometry[19] using absorption data for purified HBA solution (1–8 mM of **1** in 0.1 M Tris at pH 7.5, 0.05 mm path length).

4.3. UV irradiation to induce DNA lesion

DNA solution (1 mL, 10 μg/mL in 10 mM phosphate buffer, pH 7.5) in a plastic petri dish (35 mm ϕ. Asahi Techno Glass Corporation, Tokyo, Japan) was covered with a square quartz cell (4 cm × 4 cm, path length 0.1 mm) filled with anthocyanin solution (5 mM in 100 mM phosphate buffer, 0.5% TFA aqueous or methanol containing 0.5% TFA). From the upper side, monochromatic UV light with the Okazaki large spectrograph[23] or broad UV-B light with UV-B lamps (FL20SE, Toshiba, Tokyo, Japan) was irradiated. When the OLS was used, a neutral density filter (ND filter) was placed on the square quartz cell to equilibrate its energy with a PFDM-200LX dosimeter (Rayon Industrial, Kawasaki, Japan). When UV-B lamps were used, the cell was covered with a UV-29 filter (light transparency at 260, 290, and 340 nm of 10%, 50% and 90%, respectively, Toshiba Glass, Tokyo, Japan) to cut off UV-C light and the energy level reached in the DNA solution was recorded using a UV digital radiometer (UV-103, Macam Photometrics Ltd., Scotland, UK) at 310 nm. DNA solution was irradiated through a quartz cell filled with solvent without any pigment as the control.

4.4. Quantification of DNA damage

The amounts of CPDs and 6-4PPs in irradiated DNA were quantified by ELISA according to the protocol described by Mori et al. with modification.[24] Fifty microliter aliquots of diluted DNA solution (10–15 ng/well for CPDs, 150–350 ng/well for 6-4PPs with 10 mM phosphate buffer saline (PBS), pH 7.4) were poured into 96-well plates pre-coated with 1% protamine sulfate (50 μL/well) and incubated at 45 °C for 12 h. After drying, the plates were washed 5 times with PBS containing 0.05% Tween 20 (PBS-T). The plates were incubated with 1% newborn calf serum in PBS (200 μL/well) at 37 °C for 90 min and washed again. One hundred microliters of specific antibody (1/1000 in PBS) for CPDs (TDM-2) or 6-4PPs (64M-2) were then added to each well with incubation at 37 °C for 90 min. The plates were washed 5 times with PBS-T and then incubated with 100 μL of goat anti-mouse IgG(H + L) conjugated with biotin, F(ab′)$_2$ fragment (1/1000 in PBS, Zymed, San Francisco, CA) at 37 °C for 90 min. After the plates were washed 5 times with PBS-T, 100 μL of streptavidin conjugated with horseradish peroxidase (1/10,000 in PBS, Zymed) was added to the wells and the plates were incubated at 37 °C for 90 min. Finally, after three washings with PBS-T and two subsequent washings with citrate–phosphate buffer (pH 5.0), 100 μL of substrate solution, consisting of 0.04% *o*-phenylenediamine and 0.007% H_2O_2 in citrate–phosphate buffer, was added to each well. Following 30 min incubation at 37 °C, 50 μL of 2 M H_2SO_4 was added to stop the reaction and the absorbance at 490 nm was measured using an Immuno Reader NJ-2000 (Japan Spectroscopic Co., Tokyo). All the assays were performed in triplicate. The effects of anthocyanins were calculated as compared to the controls.

4.5. Stability of HBA and trisdeacyl HBA under solar radiation

A quartz cell (path length 0.1 mm) filled with anthocyanin solution (5 mM in 100 mM phosphate buffer, pH 7.5) was covered with a UV-29 cut filter (Toshiba

Glass). The cell was set outside and exposed to solar radiation in November 2004 at Nagoya city. The UV-B dose was monitored by UV Monitor MS-211-I with a UV-B sensor (EKO Instruments, Tokyo) covered with a UV-29 cut filter. The amount of the anthocyanins was quantified by HPLC analysis under the following conditions: column: Develosil ODS-HG-5 (2 mm $\phi \times 250$ mm), solvent: 0.5%TFA–19% aq CH_3CN (for HBA) or 0.5%TFA–14% aq CH_3CN (for trisdeacyl HBA), temperature: 40 °C, detection: 530 nm.

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