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# Physiological Roles of Betacyanin in a Halophyte, *Suaeda japonica* Makino

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**Abstract:** Halophytes can be used as a crop on salinized agricultural land. *Suaeda japonica* Makino is an annual highly salt-tolerant plant, that inhabits salt marshes in the Ariake Sea, Japan. Accompanying growth, leaves of *S. japonica* change from green to red with accumulation of a red pigment, betacyanin. To elucidate the physiological roles of betacyanin in *S. japonica*, we tested the antioxidant capacity of purified betacyanin and leaf extracts containing different levels of betacyanin under oxidative-stress conditions. The assay with 1,1-diphenyl-2-picrylhydrazyl (DPPH) showed that the purified betacyanin had antioxidant activity. H<sub>2</sub>O<sub>2</sub>-induced protein oxidation of the leaf extracts was prevented by the addition of betacyanin. The antioxidant enzyme activity decreased in the red leaves, but the content of malondialdehyde (MDA), an oxidative stress marker in the red leaves was nearly the same as that in the green leaves. Betacyanin synthesis was induced under photoinhibition-inducible conditions of low temperature and high-intensity light. These results indicate that betacyanin serves as an antioxidant in *S. japonica* and that the leaf reddening of this species is a key adaptive strategy for coping with the harsh environmental conditions in salt marshes of the Ariake Sea.

**Key words:** Antioxidant, Betacyanin, Chlorophyll, Halophyte, Reactive oxygen species, *Suaeda japonica* Makino.

Salinization is an important limiting factor for sustainable agriculture in modern agricultural settings. Halophytes have been utilized as a vegetable, forage and oilseed crop (Glenn et al., 1999) and as a cleaning crop (Ravindran et al., 2007), and have been evaluated as a potentially useful crop resource on saline soils. There are many halophytes inhabiting the Ariake Sea in Japan, but their characteristics and utilization have not been elucidated well. *Suaeda japonica* Makino is an annual hydrohalophyte belonging to the Chenopodiaceae, and inhabits the salt marsh of the Ariake Sea. *S. japonica* is exposed to severe environmental conditions such as flooding at high tide and dehydration at ebb tide. Thus, *S. japonica* is expected to have unique adaptive strategies to survive in harsh conditions, but only a few studies on the mechanisms of stress tolerance in this species have been reported. Yokoishi and Tanimoto (1994) and Tanimoto et al. (1997) showed that the synthesis of glycinebetaine was up-regulated under salt stress conditions in the seedlings and callus tissues of *S. japonica*.

Glycinebetaine is a well-known compatible solute, and

increases salt tolerance through accumulation of glycinebetaine in a wide variety of halophytic plants, and the genes responsible for glycinebetaine production (e.g., choline mono-oxygenase and betaine aldehyde dehydrogenase) were isolated and characterized (Moghaieb et al., 2004).

A distinctive trait of *S. japonica* is its change of leaf color at different growth stages. Leaf reddening, due to the accumulation of betacyanin, occurs in young seedlings in early spring and in adult plants at reproductive growth stage in autumn. The betacyanin degrades and chlorophyll is synthesized during the vigorous growth stage in summer. In early spring and autumn, a community of *S. japonica* is composed of plants with green, green-red and red leaves. Chlorophyll synthesis in the red leaves is also easily induced by nitrogen fertilizer. Therefore, the leaf reddening seems to proceed in the normal course of shoot ontogeny, and is induced, in part, by environmental stimuli. However, the development and environmental basis for the induction of betacyanin production have not been clarified.

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**Abbreviations:** APX, ascorbate peroxidase; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; GSSG, oxidized glutathione; GR, glutathione reductase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

Betacyanins together with betaxanthins belong to a class of nitrogenous chromoalkaloids known as betalains. Anthocyanins are widely distributed in higher plants, but betacyanins accumulate in only ten families in the order Caryophyllales (e.g., Amaranthaceae, Cactaceae, and Chenopodiaceae etc.). In these species, betacyanins replaced anthocyanins, and these pigments are not found together in the same plant (Stafford, 1994).

Although the accumulation of anthocyanins and betacyanins is mutually exclusive in plants, similar characteristics indicate a functional analogy between the two groups of pigments. The production of anthocyanins can be induced under abiotic stresses conditions such as high irradiance, temperature extremes, UV radiation, nutrient deficiencies, dehydration and mechanical injury, as well as biotic stresses such as herbivory and pathogen attack (Gould, 2004). The production of betacyanin has also been reported to be induced under UV-A light exposure (Vogt et al., 1999), mechanical lesion and inoculation of pathogenic fungi (Sepúlveda-Jiménez et al., 2004). These same stressors lead to the generation of reactive oxygen species (ROS), indicating that betacyanin serves as an antioxidant to alleviate oxidative damage in the cells of *S. japonica*.

*In vitro* experiments have shown that anthocyanins have potent antioxidant capacities, and protect against oxidative injury in leaves by scavenging reactive oxygen species (Gould et al., 2002). Similarly, betalains serve as free radical scavengers and prevent active oxygen-induced and free radical-mediated oxidation of biological molecules (Pedreño and Escribano, 2001). For instance, betanin and betanidin extracts from *Beta vulgaris* inhibited lipid peroxidation caused by metmyoglobin or lipoxygenase activity, the peroxidation of microsomes from turkey muscle tissue, and the oxidation of low-density lipoproteins (LDL) of human blood (Kanner et al., 2001). In this study, to examine the physiological significance of betacyanin in *S. japonica*, we measured the antioxidant activity of betacyanin. Our results clearly showed that betacyanin of *S. japonica* has high antioxidant activity, and it accumulated under the conditions that induce photoinhibition of photosynthesis.

### Materials and Methods

Leaf tissues of *S. japonica* used for the measurement of seasonal changes in pigments were collected from plants growing along the shoreline of the Ariake Sea. Fully expanded leaves were collected from the top 2/3 portion of shoot and were immediately immersed in liquid nitrogen, and the frozen samples were stored at -80°C until analysis. We selected a community that was composed of plants with different levels of betacyanin. In April, leaves with different degrees of red-coloration categorized into green, green-red and red were collected from plants in a 5

m×15 m plot within a single community of *S. japonica*. Plants about 15 cm in height were harvested. The leaf tissues were collected and stored as described above. For assay of protein oxidation, plants were cultured in 10 L containers containing Hoagland's solution with aeration. The leaves of three-month-old plants were collected and preserved as described above. For experiments of betacyanin induction, the plants grown in the community of plants with green leaves were collected from the shoreline, and transferred to a growth chamber (KOITOTRON, KG-50HLA), and were cultured at different temperature (day/night: 25°C/20°C and 10°C/10°C) and light intensity (200–300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 1300–1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions described in the text. The leaf tissues were collected and stored as described above.

### 1. Extraction and quantification of pigments

Betacyanin and chlorophyll were extracted in water and acetone, respectively. No contamination of chlorophyll with betacyanin or *vice versa* was observed in the extract. To extract chlorophyll, we homogenized the frozen leaf samples in 100% acetone. 2.5 mM Na-phosphate buffer (pH 7.8) was added to the extract to adjust the acetone concentration to 80%, and absorbance of extract was measured at 663.6 nm and 646.6 nm with a spectrophotometer (Shimadzu, UV-160A). The concentration of chlorophyll was calculated by the formulas of Porra et al. (1989). The remaining precipitation was dried by evaporating in the dark at 4°C with a rotary evaporator (Yamato, RD400) and re-suspended in deionized water. After centrifugation at 17,000×g for 30 min at 4°C, the absorbance of supernatants was measured at 538 nm. Betacyanin concentrations were determined using a molecular extinction coefficient of 60,000 L mol<sup>-1</sup> cm<sup>-1</sup> (Stüntzing et al., 2003). Betacyanin was purified by the method of Wang et al. (2006) with minor modification. Frozen leaves were homogenized in 10 volumes of methanol for 30 min at 4°C and were centrifuged at 17,000×g for 15 min at 4°C. The supernatant was discarded, and the pellet was re-extracted in 10 volumes of ethanol and was centrifuged at 17,000×g for 15 min at 4°C. This procedure was repeated to remove chlorophyll, carotenoid, ascorbic acid and tocopherol to extract betacyanin, the pellet was then re-extracted with 50% of ethanol for 30 min, and was centrifuged at 17,000×g for 15 min at 4°C. The supernatant was mixed with a mixture containing phenol, chloroform and isoamyl alcohol (25:24:1), and centrifuged at 17,000×g for 15 min at 4°C to remove protein. The supernatant was extracted with a mixture of chloroform and isoamyl alcohol (24:1) to remove phenol. The concentration of purified betacyanin was determined spectrophotometrically by the same procedure as described above.

## 2. Extraction of proteins

Frozen samples of leaves (0.5 g) were homogenized in 2.0 mL of extraction buffer containing 50 mM Naphosphate buffer (pH 7.5), 0.5% polyvinylpyrrolidone, 0.1% Triton X-100 and 3 mM EDTA. The homogenate was centrifuged at  $17,000\times g$  for 5 min at 4°C. The supernatant was used for assay of antioxidant enzyme activities. The protein content in the supernatant was determined by the method of Bradford (1976) using bovine serum albumin as a protein standard.

## 3. Antioxidant enzyme activity

Superoxide dismutase (SOD) activity was measured according to the method of Beauchamp and Fridovich (1971). The reaction mixture contained 40 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75  $\mu$ M nitro blue tetrazolium (NBT), 2  $\mu$ M riboflavin and 0.1 mM EDTA. The crude extract was added to the mixture and placed under 15-W fluorescent lamp for 30 min. The absorbance of the reaction mixture was measured at 560 nm with the above spectrophotometer. One unit of activity was expressed as the amount of fresh weight required to inhibit 50% of the initial reduction of NBT under the light.

Ascorbate peroxidase (APX) activity was measured according to the method of Nakano and Asada (1981). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 0.2 mM  $H_2O_2$  and 0.5 mM sodium ascorbate. The assay was carried out at 25°C. The change in absorbance of the reaction mixture was monitored at 290 nm with the above spectrophotometer. Enzyme activity was calculated using the molar extinction coefficient of ascorbate ( $2.8 \text{ L mmol}^{-1} \text{ cm}^{-1}$ ).

Catalase (CAT) activity was measured as the disappearance of  $H_2O_2$  at 240 nm with the above spectrophotometer according to the methods of Beers and Sizer (1952) and Nelson and Kiesow (1972) with minor modification. The reaction mixture contained 25 mM Tris-HCl buffer (pH 7.8) and 10 mM  $H_2O_2$ . The assay was carried out at 25°C. Enzyme activity was calculated using the molar extinction coefficient of  $H_2O_2$  ( $39.4 \text{ L mmol}^{-1} \text{ cm}^{-1}$ ).

Glutathione reductase (GR) activity was determined by measuring NADPH oxidation (Foyer and Halliwell, 1976). The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.8), 2 mM EDTA, 50  $\mu$ M NADPH and 0.5 mM GSSG. The assay was carried out at 25°C. The change in absorbance of reaction mixture was monitored at 340 nm with the above spectrophotometer. Enzyme activity was calculated using the molar extinction of NADPH ( $6.22 \text{ L mmol}^{-1} \text{ cm}^{-1}$ ).

## 4. Radical scavenging activity

Frozen samples of leaves were extracted by the method of Pavlov et al. (2002) with minor modification. A free

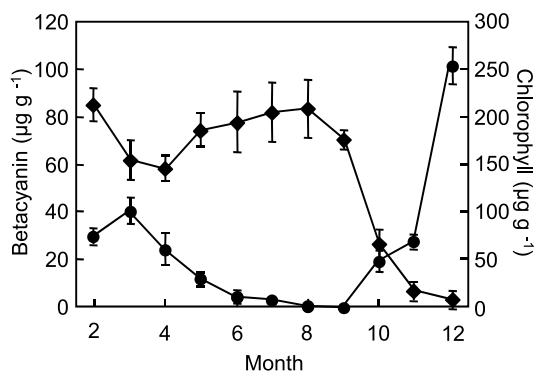


Fig. 1. Seasonal changes in betacyanin (●) and chlorophyll (◆) contents in leaves of *S. japonica*. The error bars indicate standard error;  $n=8-10$ .

radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was assessed according to the method of Ansari et al. (2005). Frozen leaves were homogenized in 50% ethanol, and the homogenate was centrifuged at  $3,000\times g$  for 10 min at 4°C. MES buffer (pH 6.0) and ethanol were added to the resultant supernatants to adjust the concentration to 100 mM and 50%, respectively. The extract was added to an equal volume of 0.1 mM DPPH, and incubated at room temperature under darkness for 5 min. Absorbance of mixture was measured at 518 nm with the above spectrophotometer. The free radical scavenging activity was calculated as described by Ansari et al. (2005).

## 5. Lipid peroxidation

Lipid peroxidation was determined by malondialdehyde (MDA) content according to the method of Hodges et al. (1999). The concentration of thiobarbituric acid reactive substances was calculated by using the molar extinction coefficient of MDA ( $157 \text{ L mmol}^{-1} \text{ cm}^{-1}$ ).

## 6. Protein oxidation induced by hydrogen peroxide

Proteins were extracted according to the method of Prasad (1996). Leaf tissue (0.5 g) was ground in 5 mL extraction buffer containing 0.1 M K-phosphate buffer (pH 7.0), 1 mM  $Na_2$ -EDTA, 1 mM PMSF, 2.5  $\mu$ g leupeptin and 2.5  $\mu$ g aprotinin. The homogenate was centrifuged at  $12,000\times g$  for 15 min at 4°C. Hydrogen peroxide ( $H_2O_2$ ) was added to the supernatant to reach the final concentrations of 0.1% and 0.2%. Betacyanin was added to the mixture at concentration of 1.0, 2.0 and 3.9  $\text{mg L}^{-1}$ . After incubation in the dark at 25°C for 1 hour, protein oxidation was evaluated by carbonyl content according to the method of Levine et al. (1990) and Prasad (1996). The protein was precipitated by 10% trichloroacetic acid (TCA) and centrifuged at  $12,000\times g$  for 5 min at 4°C. The pellet was dissolved in 10 mM 2,4-dinitrophenylhydrazine or 2 M HCl (control) for 1 hour at room temperature. The proteins were precipitated with TCA as described above





Fig. 2. Three phenotypic categories of leaf colors in *S. japonica* in April. (A), Green; (B), Green-red; (C), Red. The scale bars show 10 mm.

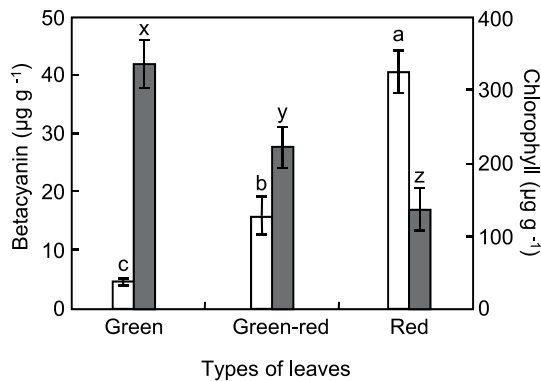


Fig. 3. Betacyanin (□) and chlorophyll (■) contents in *S. japonica* leaves with different leaf colors in April. The error bars indicate standard error; n=5. Different letters indicate a statistically significant difference among the leaves (Scheffe's F test,  $P < 0.05$ ).

and the pellet was washed with a mixture of ethanol and ethyl acetate (1:1). The final protein pellets were dissolved in 6 M guanidine (pH 2.3) and the absorbance of solution was measured at 360 nm with the above spectrophotometer. The carbonyl content was calculated using the extinction coefficient of hydrazone ( $22,000 \text{ L mol}^{-1} \text{ cm}^{-1}$ ).

## Results

Fig. 1. shows seasonal changes in the content of betacyanin and chlorophyll in *S. japonica* leaves. Betacyanin showed a transient accumulation in the leaves of seedlings in March, a gradual decline up to September, followed by peak accumulation in December. Chlorophyll content changed inversely with betacyanin. Chlorophyll content was high in February, declined gradually through April, was high during the summer months, but then declined during senescence in late autumn. The communities of *S. japonica* were composed of plants with green, green-red and red leaves in April and May.

Fig. 2. shows the three phenotypic categories, green (A), green-red (B) and red (C) of *S. japonica* in April. The

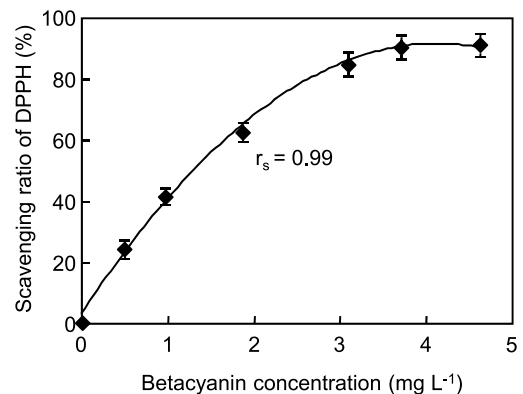


Fig. 4. DPPH radical scavenging activity of purified betacyanin. The error bars indicate standard error; n=4.  $r_s$  is Spearman's rank correlation coefficient.

leaves of plants grown at a high density turn red, whereas the leaves of plants grown at a low density remain green. The plants used in the present study were selected according to their leaf colors when grown in a single community.

Fig. 3. shows the betacyanin and chlorophyll contents of leaves of *S. japonica*. The betacyanin content per g fresh weight of green, green-red and red leaves were about 4, 16, and 40  $\mu\text{g}$ , respectively. The content of betacyanin and chlorophyll mirrored their visual appearance.

Fig. 4. shows the antioxidant capacity of betacyanin purified from *S. japonica* leaves. The DPPH radical scavenging activity increased with the betacyanin level. The betacyanin concentration was found to be positively correlated with the intensity of the antioxidant capacity ( $r_s = 0.99$ ).

Purified betacyanin reduced  $\text{H}_2\text{O}_2$ -induced protein oxidation in crude leaf extracts evaluated as the amounts of carbonyl compounds generated in amino acid side chains, which was induced by  $\text{H}_2\text{O}_2$  (Fig. 5). In the control, the carbonyl content was 27 nmol per mg protein, and in the leaves treated with 0.1% and 0.2%  $\text{H}_2\text{O}_2$ , it was 37 nmol and 47 nmol, respectively (Fig. 5A). The degree of protein oxidation increased with the  $\text{H}_2\text{O}_2$  level. However,

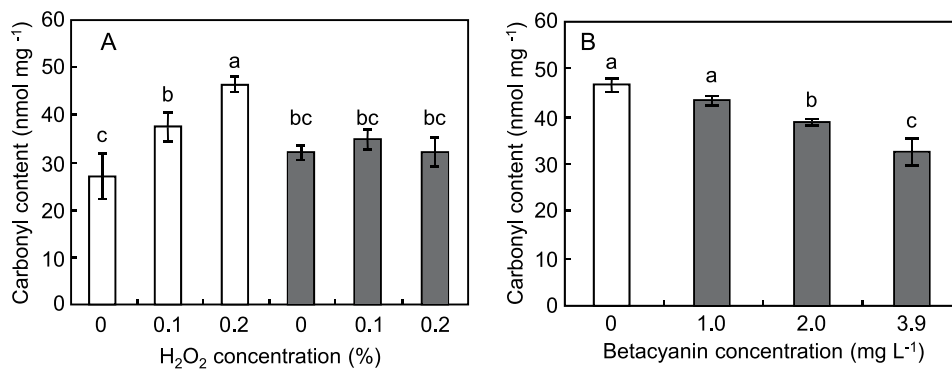


Fig. 5. Effect of betacyanin on protein oxidation in leaf extract. Carbonyl content was measured in the leaf extract with (□) and without betacyanin (■). Betacyanin (3.9 mg L<sup>-1</sup>) alleviated H<sub>2</sub>O<sub>2</sub>-induced protein oxidation (A). Protein oxidation induced by 0.2% H<sub>2</sub>O<sub>2</sub> decreased with increase of concentration of betacyanin in the extract (B). The error bars indicate standard error; n=4. Within each figure, different letters indicate a statistically significant difference among treatments (Scheffe's F test, P<0.05).

in the presence of betacyanin, the level of oxidized protein in extracts treated with 0.1% and 0.2% H<sub>2</sub>O<sub>2</sub> was similar to that in the extract without H<sub>2</sub>O<sub>2</sub>-treatment (Fig. 5A). The degree of protein oxidation in leaf extract induced by 0.2% H<sub>2</sub>O<sub>2</sub> was reduced by the addition of betacyanin (Fig. 5B).

Fig. 6. shows the activities of antioxidant enzymes in leaves containing different levels of betacyanin expressed on a fresh weight basis. The activity of SOD, which catalyzes superoxide anion into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>, was nearly the same among the leaves containing different levels of betacyanin. However, the activities of APX, which catalyzes H<sub>2</sub>O<sub>2</sub> into H<sub>2</sub>O, CAT, which decomposes H<sub>2</sub>O<sub>2</sub>, and GR, which catalyzes the reduction of oxidized glutathione, all declined with increasing betacyanin content.

The antioxidant capacity (evaluated by DPPH radical scavenging activity) of crude extract in green, green-red and red leaves was about 58%, 46% and 45%, respectively (Fig. 7). The overall activity was slightly higher in the green leaves than in the more red-colored leaves, but the difference in antioxidant capacity was smaller than that in the activity of antioxidant enzymes (Fig. 6). The antioxidant capacity of betacyanin purified from the crude extracts was higher in the red leaves, which reflected the content of betacyanin in the crude extracts. The antioxidant capacity of purified betacyanin in the green, green-red and red leaves was 3%, 8% and 14%, respectively. The proportions of radical scavenging capacity of betacyanin as a percentage of total antioxidant capacity

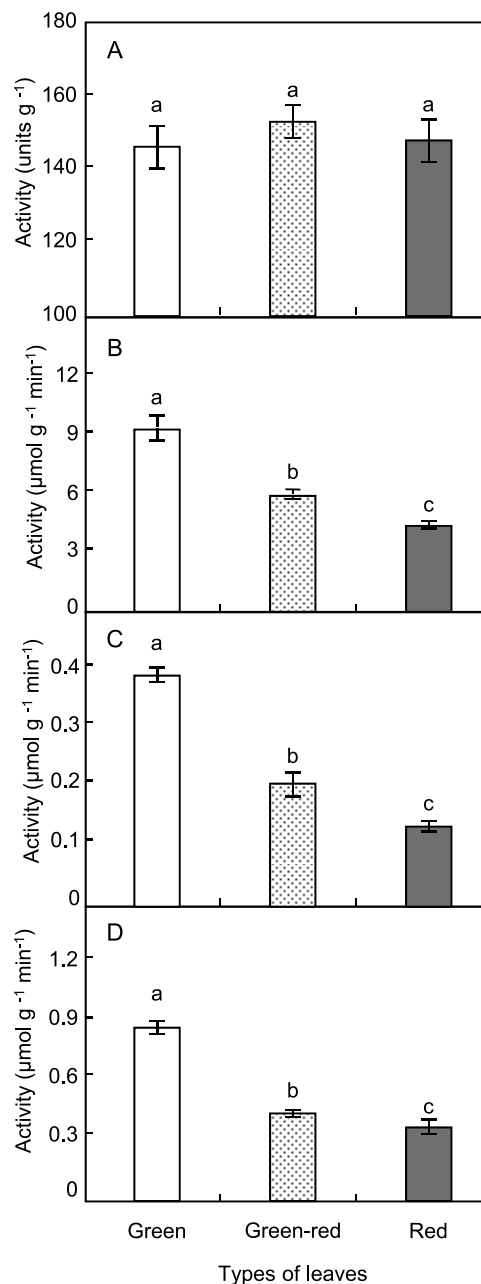


Fig. 6. Antioxidant enzymes activity in leaves containing different levels of betacyanin. (A), SOD; (B), APX; (C), CAT; (D), GR. The error bars indicate standard error; n=4. Within each figure, different letters indicate a statistically significant difference among the leaves (Scheffe's F test, P<0.05).

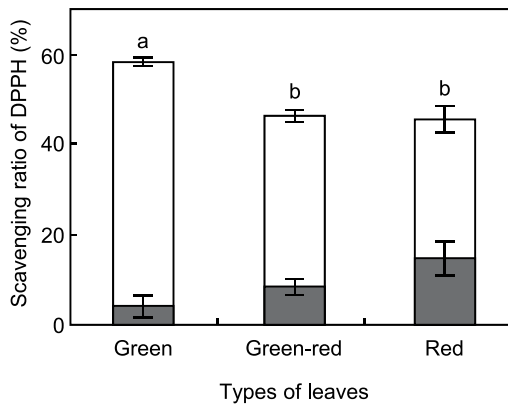


Fig. 7. Antioxidant capacity expressed by scavenging of DPPH in crude extracts of leaves and purified betacyanin solution. Percentage of antioxidant capacity of betacyanin (shaded bar) to total antioxidant capacity (open bar) in the leaves containing different levels of betacyanin. The error bars indicate standard error;  $n=4$ . Different letters indicate a statistically significant difference among the leaves (Scheffe's F test,  $P<0.05$ ).

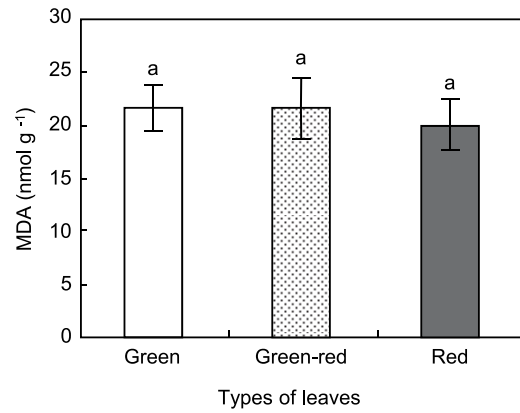


Fig. 8. Malondialdehyde content in leaves containing different levels of betacyanin. The error bars indicate standard error;  $n=4$ . Different letters indicate a statistically significant difference among the leaves (Scheffe's F test,  $P<0.05$ ).

of green, green-red and red leaves were 5%, 17% and 30%, respectively, (Fig. 7). Thus the contribution of betacyanin to antioxidant capacity was highest in the red leaves.

MDA, an index of oxidized unsaturated fatty acid and an oxidative stress marker in cells was measured in the leaves containing different levels of betacyanin (Fig. 8). The MDA content of green, green-red and red leaves were 21.6, 21.7 and 20.0 nmol per g fresh weight basis, respectively. There was no statistically significant difference in MDA among these leaves, indicating that the level of the oxidative stress was almost the same among the leaves containing different levels of betacyanin.

Fig. 9. shows the effects of temperature and light intensity on the betacyanin and chlorophyll contents of leaves of *S. japonica*. Betacyanin content increased with the marked decrease in chlorophyll content after the start of exposure of plants to a combination of low temperature (day/night: 10°C/10°C) and high-intensity light (1300–1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Betacyanin content increased linearly with duration of treatment, whereas chlorophyll content decreased at 20 days after treatment to about 100  $\mu\text{g}$  per g fresh weight and was maintained at this level thereafter.

### Discussion

There is little physiological explanation for the presence of betacyanin in the leaves of plant species in Centrospermae; especially in *S. japonica*, no attempt has been made to elucidate the roles of betacyanin.

In plants that accumulate anthocyanin, pigmentation is induced by environmental stresses (Chalker-Scott, 1999), and red leaves appear at predictable times of the year and at specific stages in leaf development. These observations

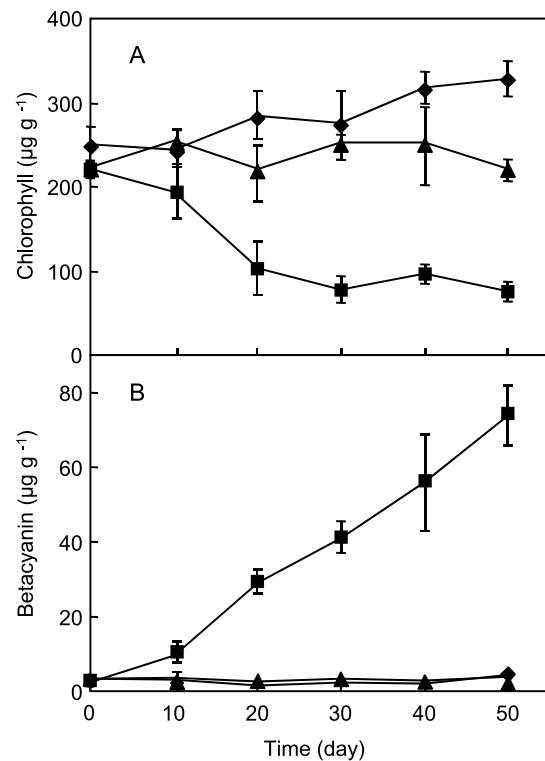


Fig. 9. Chlorophyll (A) and betacyanin (B) contents in *S. japonica* under different temperature and light conditions. (◆), 25°C/20°C, 200–300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; (▲), 10°C/10°C, 200–300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; (■), 10°C/10°C, 1300–1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The error bars indicate standard error;  $n=5$ .

prompted researchers to postulate hypotheses in physiological function to account for the presence of anthocyanins in leaves, including (i) protection of tissues against harmful effects of UV-B irradiation (Burger and Edwards, 1996; Klaper et al., 1996), (ii) modification of the quantity and quality of absorbed light (Lee, 1986; Barker et al., 1997), (iii) ROS scavenging (Yamasaki, 1997; Sherwin

and Farrant, 1998), and (iv) protection of photosynthetic apparatus against photoinhibition (Gould et al., 1995; Dodd et al., 1998). Similarly, betacyanin production was induced by abiotic and biotic stresses, which potentially cause ROS production. In *S. salsa*, a halophytic species belonging to the same genus as *S. japonica*, betacyanin synthesis was induced by low temperature, and high salinity (Wang et al., 2006). In *Mesembryanthemum crystallinum* betacyanin synthesis was induced by UV-A (Vogt et al., 1999). In *S. japonica* betacyanin accumulated when temperature dropped significantly in February to March and November to December (Fig. 1). These observations indicate that betacyanin has a function similar to anthocyanin.

Betacyanin purified from *S. japonica* leaves had radical scavenging activity evaluated by DPPH assay (Fig. 4), and reduced H<sub>2</sub>O<sub>2</sub>-induced protein oxidation (Fig. 5). The activities of the antioxidant enzymes (APX, CAT and GR) decreased in the leaves containing betacyanin (Fig. 6), but the total radical scavenging capacity of leaf extracts evaluated by DPPH has not hardly decreased in the red leaves (Fig. 7). The contribution of betacyanin to total radical scavenging activity was higher in the red leaves than in the leaves that contain only small amounts of betacyanin (Fig. 7). In addition, the lipid peroxidation evaluated by MDA content was almost the same in leaves containing different levels of betacyanin (Fig. 8). These results suggested that betacyanin acts as a ROS scavenger. Betacyanin and anthocyanin accumulated mainly in the vacuole (Tanaka et al., 2008), and anthocyanin-rich vacuole has been shown to be a potential sink for excess H<sub>2</sub>O<sub>2</sub> produced in the chloroplast, alleviating the photo-oxidative risk (Kytridis and Manetas, 2006). Generation of superoxide anion radicals in the chloroplast is normally reduced via the action of SOD to H<sub>2</sub>O<sub>2</sub>, which is then decomposed by CAT or APX. If in excess or not properly handled, the activated oxygen can further generate hydroxyl radicals which attack proteins and lipids, causing radical chain reactions. Hydroxyl radicals cannot penetrate the tonoplast and, accordingly, have to be scavenged in the chloroplast or the cytoplasm (Takahashi and Asada, 1983). Yet, H<sub>2</sub>O<sub>2</sub> can freely diffuse into the vacuole where anthocyanin and betacyanin abound (Yamasaki et al., 1997). APX has been shown to be the primary target of photo-oxidative stress in leaves treated with a ROS inducer methyl viologen (Mano et al., 2001). Inactivation of APX precedes that of SOD under photo-oxidative stress treatment (Mano et al., 2001), and thus H<sub>2</sub>O<sub>2</sub> transiently accumulates in the chloroplast stroma (Nakano and Asada, 1981), and further diffuses to other cell compartments under photo-oxidative conditions. Anthocyanin in the vacuole has been suggested to function reducing the ROS (Kytridis and Manetas, 2006). Similarly, in *S. japonica*, APX and CAT activities were lower in the red leaves, than in the

green leaves while SOD activity was maintained at nearly the same level (Fig. 6). The maintenance of SOD activity in red leaves was suggested to permit betacyanin to function efficiently as an antioxidant to scavenge H<sub>2</sub>O<sub>2</sub>.

In the autumnal leaves of plant species that accumulate anthocyanin, the pigment synthesis often has been reported to precede chlorophyll breakdown and pigment accumulation in senescing leaves is increased by high-intensity light, cool temperatures, and mild drought (Wheldale, 1916; Kozłowski and Pallardy, 1997; Dodd et al., 1998; Chalker-Scott, 1999), coinciding with increased photoinhibitory risk. In *S. japonica*, betacyanin accumulation was induced by exposure to combining low temperature and high-intensity light (Fig. 9), which is a well-known environmental condition that makes plants more vulnerable to photoinhibition (Murata et al., 2007). Under this condition, betacyanin increased almost linearly with the duration of treatment, whereas chlorophyll content decreased 20 days after treatment to about half of the control level, and was maintained at this level thereafter (Fig. 9). Our previous study, also suggested that betacyanin alleviated ROS-induced oxidative damage in biological molecules in the leaves of *S. japonica*. The degradation of chlorophyll by methyl viologen was ameliorated in the red leaves (Hayakawa et al., 2008). The maintenance of chlorophyll content in the red leaves may be due to photoprotection provided by betacyanin acting as a ROS scavenger under hyperoxidant conditions. A positive correlation ( $r=0.88$ ) between photosynthetic O<sub>2</sub> evolution rate and chlorophyll content has been found in *S. japonica* (Hayakawa et al., 2008), indicating that photosynthetic activity would be maintained in nearly the same pattern as chlorophyll content in the red leaves under photo-oxidative stress-inducible conditions.

In conclusion, the seasonal changes in betacyanin accumulation, a distinguishing feature of *S. japonica*, was found to be important for the amelioration of ROS-induced photoinhibition and related photo-oxidative stresses. The accumulation of betacyanin is likely to be an adaptation strategy that allows this hydrohalophyte to survive under the harsh environmental conditions in the tidal flat along the shoreline of the Ariake Sea.

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\* In Japanese with English abstract.