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Photoprotective roles of anthocyanins in *Begonia semperflorens*

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ABSTRACT

Anthocyanins are thought to provide photoprotection under stressful conditions. We used two genotypes of *Begonia semperflorens* with different pigmentation to study the effects of anthocyanins on tolerance of high-light stress. The maximum quantum yield of PSII (Fv/Fm) in red leaves was significantly higher than that in green leaves during and after high-light stress. High light also induced significant increases in anthocyanins in both genotypes. Despite large differences in anthocyanin content, chlorophyll content and Chl a/b ratio did not differ between red and green leaves. After high-light stress, xanthophyll pool size and enzymatic antioxidant activity were lower in red leaves than in green leaves. Non-enzymatic antioxidant activity measured by DPPH assay, however, was significantly higher in red leaves than in green leaves. Meanwhile, changes in DPPH activity were closely correlated with changes in anthocyanin content during and after high-light stress. Declines in Fv/Fm were significantly greater for green leaves than for red leaves under green light but were comparable between green and red leaves under red and blue light. Changes in ROS under different colors of light were similar to the changes in Fv/Fm. Our results suggest that anthocyanins primarily function as light filters rather than as antioxidant molecules during high-light stress in *B. semperflorens*.

1. Introduction

Under natural conditions, plants are frequently exposed to environmental fluctuations, especially in light intensity. High light levels that exceed the utilization capacity of plants cause a series of events that can ultimately lead to destruction of the photosynthetic apparatus of exposed leaves. The key event in oxidative stress is the photogeneration of reactive oxygen species (ROS), such as $^1O_2$, $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$, following the formation of chlorophyll triplets in the light-harvesting antennae when plants absorb excessive light [1]. To combat photooxidative stress, plants are equipped with a diverse set of photoprotective processes including strategic leaf and chloroplast movements, non-radiative dissipation of absorbed excitation energy, detoxification of chloroplast ROS via intricate antioxidant pathways, repair processes to prevent photodamages, and utilization of excess absorbed light by an array of alternative electron acceptors [2].

Non-photochemical quenching (NPQ) is an important photoprotective mechanism that is induced within seconds to minutes upon exposure to excess light [3]. This process dissipates excess absorbed energy as heat, minimizing the excitation pressure on photosystem II (PSII). NPQ is known to depend on three factors: the PsbS protein, the xanthophyll zeaxanthin and the establishment of a pH gradient across the thylakoid membrane [4]. While PsbS is involved specifically in NPQ, zeaxanthin may play a dual role. It may cause quenching of excitation energy, and it has been shown to be an important membrane antioxidant [5,6]. When light intensity is high enough to saturate NPQ, direct reduction of O_2 at PSI could be an effective strategy to reduce excitation pressure [7,8]. However, this process causes the formation of hazardous ROS in the chloroplast. Plants have evolved antioxidant enzymes to scavenge ROS in the photosynthetic apparatus. The $O_2^{\bullet -}$ generated by the direct reduction of O_2 in the vicinity of photosystem I (PSI) is rapidly converted to H_2O_2 by superoxide dismutase (SOD), while H_2O_2 is detoxified by catalase (CAT) in the peroxisome and by ascorbate peroxidase (APX) in the chloroplast. This reaction is accompanied by the production of oxidized ascorbate, which is regenerated by dehydroascorbate reductase (DHAR) in the presence of glutathione [7].

Like flowers, the leaves of many ornamental plants exhibit a wide variety of colors, such as green, blue and red. Leaf color is an important attribute for marketability and consumer preference. Anthocyanins, which are located in upper epidermis, palisade layers, and lower epidermis, are primarily responsible for red-to-blue

Abbreviations: A, antheraxanthin; APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; Fm, maximal fluorescence; Fo, minimal fluorescence; Fv/Fm, maximum quantum yield of PSII; NPQ, non-photochemical quenching; POD, peroxidase; PPFD, photosynthetic photon flux density; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; V, violaxanthin; Z, zeaxanthin.

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leaf coloration [9]. However, the physiological roles of vegetative pigmentation are less clearly understood. Anthocyanins are often produced in vegetative tissues under stressful conditions, such as high light, cold temperatures, nutrient deficiency and pathogen attack [10,11]. Previous studies have presented evidence for the ability of anthocyanins to provide photoprotection under stressful conditions. There is evidence that anthocyanins protect the photosynthetic apparatus from photoinhibition by absorbing green light and thereby reducing excess excitation energy [12–15]. On the other hand, anthocyanins, which belong to one class of polyphenols, may serve as antioxidants due to their unique chemical structure [16–19]. Therefore, anthocyanins may play a photoprotective role by directly eliminating ROS during photooxidative stress. However, not all studies have found strong relationships between anthocyanins and photoprotection. Burger and Edwards [20] found no difference in photoinhibition between red and green *Coleus* varieties exposed to photoinhibitory treatment. There is even some evidence for increased susceptibility to photoinhibition in anthocyanic leaf areas [21]. In addition, one should also consider that the differential absorption of light wavelength in red and green leaves may lead to differential acclimation to high light, and thus the effects of light shielding could potentially be mixed with those of acclimation.

Begonia semperflorens is one of the most popular bedding plants. Depending on the variety, leaves of *B. semperflorens* may be green or red. Red-leaf genotypes grow better than green-leaf genotypes under photoinhibitory conditions during summer. In this study, we used two genotypes of *B. semperflorens*, 'Cocktail' (red leaf) and 'Super Olympia' (green leaf) to study whether differences in growth could be attributed to photoprotection offered by anthocyanins and, if so, whether this was due to the antioxidant activity or the optical properties of anthocyanins. We also analyzed the interactions between anthocyanins and other photoprotective mechanisms.

2. Materials and methods

2.1. Plant materials and treatments

The experiment was conducted in July 2009 in the greenhouse at the Institute of Vegetable Science, Zhejiang University. Seeds of two genotypes of *B. semperflorens*, the anthocyanin-accumulating 'Cocktail' (red leaf) and the low-anthocyanin-accumulating 'Super Olympia' (green leaf), were planted in 1-L plastic pots containing a mixture of peat, vermiculite and perlite (6:3:1, v/v/v). The plants were grown under shade structures constructed from PVC pipe and neutral-density cloth, providing 80% shade, in a greenhouse with day/night temperatures of 35/20 °C. 'Cocktail' and 'Super Olympia' plants grown in shaded conditions appeared red and green colors, respectively. When the plants began to bloom, they were exposed to naturally occurring high-light conditions in an open field with the maximum photosynthetic photon flux density (PPFD) being about 1500 $\mu mol\ m^{-2}\ s^{-1}$. After 3 days, plants were transferred back to shaded conditions and allowed to recover for 2 days. The maximum quantum yield of PSII (Fv/Fm) was measured at different time points after the treatment, whereas leaf samples were harvested at 0 and 3 days after the onset of high-light stress and after 2 days of recovery.

To assess the specific photoprotective effects of absorption of green light by anthocyanins, the adaxial sides of leaf disks (1.5 cm in diameter) were illuminated with red, green or blue light (660-, 522- and 452-nm in peak wavelengths, respectively) in a phytotron. The light was provided by light-emitting photodiodes (LEDs, ZDL-100 W, Nichia, Japan) as described previously [22]. The incident PPFD of each color light was maintained at about 500 $\mu mol\ m^{-2}\ s^{-1}$ by adjusting the distance from the LED lamps to the adaxial surfaces. The phytotron was kept at 10 °C in order to create photoinhibitory conditions. After 12 h of illumination, the degree of photoinhibition was evaluated by measuring the Fv/Fm and the ROS content in the leaf disks.

2.2. Chlorophyll fluorescence analyses

Chlorophyll fluorescence was measured with an imaging pulse amplitude modulated fluorometer (IMAG-MAXI; HeinzWalz, Effieltrich, Germany). Leaves or leaf disks were maintained in darkness for 20 min prior to measurement of Fv/Fm. Minimal fluorescence (Fo) was measured under a weak modulating light, and maximal fluorescence (Fm) was induced by a saturating pulse of light ($4000\ \mu mol\ m^{-2}\ s^{-1}$) applied over 0.8 s. The maximum quantum yield of PSII was determined as Fv/Fm, where Fv is the difference between Fo and Fm.

2.3. Quantification of pigments

Chlorophyll and carotenoids were extracted with 80% (v/v) acetone and measured by spectrophotometric absorption at 663 nm for Chl a, 645 nm for Chl b and 445 nm for carotenoids [23]. Anthocyanins were measured according to the methods of Pirie and Mullins [24] with some modifications. Fresh leaf tissue was ground with 5 mL of 80% ethanol and then extracted at 30 °C in a constant-temperature water bath until the green color disappeared. After centrifugation at $3500 \times g$ for 15 min, the supernatant was discarded and the pellet was homogenized with 5 mL of HCl:MeOH (1:99, v/v). The suspension was then treated with extractant at 60 °C for 60 min. To fully extract anthocyanins from the suspension, another 5 mL of extractant was sometimes used. Anthocyanin content was determined spectrophotometrically by measuring the absorbance at 530 and 600 nm. One unit of anthocyanin was defined as $(OD_{530} - OD_{600})/0.1$, and anthocyanin content was expressed as $U\ g^{-1}$ FW. Xanthophyll cycle pigments (violaxanthin, V; antheraxanthin, A; zeaxanthin, Z) were extracted with 80% acetone, filtered through a 0.45-μm membrane and analyzed by HPLC as described by Thayer and Björkman [25]. The de-epoxidation state of the xanthophyll cycle was expressed as (A + Z)/(V + A + Z).

2.4. Analysis of phenolics and flavonoids

Phenolics content was measured spectrophotometrically as described by Singleton and Rossi [26]. Frozen leaf samples (0.3 g) were homogenized with 2 mL of 80% methanol and centrifuged at $2000 \times g$ for 20 min. The reaction mixture contained 0.2 mL of supernatant and 0.15 mL of Folin-Ciocalteu's reagent. After incubation at 25 °C in darkness for 20 min, the absorbance of the reaction mixture at 735 nm was recorded. Flavonoid content was measured spectrophotometrically as described by Zhuang et al. [27]. Frozen leaf samples (0.3 g) were homogenized with 2 mL of 1% HCl:MeOH (1:99, v/v) and then centrifuged at $2000 \times g$ for 20 min. The reaction mixture contained 0.3 mL of supernatant, 0.3 mL of 5% $NaNO_2$, 0.3 mL of 10% $AlCl_3$ and 2 mL of 1 M NaOH. After reaction, the absorbance at 510 nm was recorded.

2.5. Antioxidant capacity assay

Low-molecular-weight antioxidant activity was evaluated using the stable radical α,α-diphenyl-β-picrylhydrazyl (DPPH) protocol described by Tadolini et al. [28]. Frozen leaf samples (0.3 g) were extracted with 2 mL of 80% methanol. The DPPH stock solution was prepared by stirring 75 mg of DPPH in 1 L of 80% methanol overnight. For the assay, three different mixtures were prepared, consisting of 50 μL extract + 2.5 mL DPPH solution (A),