Protective effect of supplemental anthocyanins on *Arabidopsis* leaves under high light

Xue-Qin Zeng^{a,b}, Wah Soon Chow^c, Li-Juan Su^{a,b}, Xin-Xiang Peng^d and Chang-Lian Peng^{a,b,*}

^aKey Laboratory of Ecology and Environmental Science in Guangdong Higher Education, South China Normal University, Guangzhou 510631, Guangdong, China

^bGuangdong Provincial Key Lab of Biotechnology for Plant Development, South China Normal University, Guangzhou 510631, Guangdong, China

^cDivision of Plant Sciences, Research School of Biology, College of Medicine, Biology and Environment, The Australian National University, Canberra, Australian Capital Territory 0200, Australia

^dCollege of Life Science, South China Agriculture University, Guangzhou 510642, Guangdong, China

Correspondence

*Corresponding author,
e-mail: pengchl@scib.ac.cn

Received 25 August 2009;
revised 13 October 2009

doi:10.1111/j.1399-3054.2009.01316.x

Ten anthocyanin components have been detected in roots of purple sweet potato (*Ipomoea batatas* Lam.) by high-performance liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry. All the anthocyanins were exclusively cyanidins or peonidin 3-sophoroside-5-glucosides and their acylated derivatives. The total anthocyanin content in purple sweet potato powder obtained by solid-phase extraction was 66 mg g⁻¹. A strong capacity of purple sweet potato anthocyanins (PSPA) to scavenge reactive oxygen species (superoxide, hydroxyl radical) and the stable 1,1-diphenyl-2-picrylhydrazyl organic free radical was found in vitro using the electron spin resonance technique. To determine the functional roles of anthocyanins in leaves in vivo, for the first time, supplemental anthocyanins were infiltrated into leaves of *Arabidopsis thaliana* double mutant of the ecotype Landsberg erecta (*tt3tt4*) deficient in anthocyanin biosynthesis. Chlorophyll fluorescence imaging showed that anthocyanins significantly ameliorated the inactivation of photosystems II during prolonged high-light (1300 μmol m⁻² s⁻¹) exposure. Comet assay of DNA revealed an obvious role of supplemental PSPA in alleviating DNA damage by high light in leaves. Our results suggest that anthocyanins could function in vitro and in vivo to alleviate the direct or indirect oxidative damage of the photosynthetic apparatus and DNA in plants caused by high-light stress.

Introduction

The light-mediated generation of reactive oxygen species (ROS) is inevitable in plant cells. Under strong light, the amount of ROS generated during photosynthesis

can become excessive, leading to photoinhibition and oxidative damage to the photosynthetic apparatus (Powles 1984, Shulaev and Oliver 2006). To minimize the adverse effects of ROS, plants have evolved an array of enzymatic and non-enzymatic antioxidant defences.

Abbreviations – AsA, ascorbic acid; Cy, cyanidin chloride; DAB, diaminobenzidine; DPPH•, 1,1-diphenyl-2-picrylhydrazyl; ESI, electrospray ionization; ESR, electron spin resonance; F_o , minimum fluorescence yield of a dark-adapted leaf; F_m , maximal fluorescence yield of dark-adapted leaf; F'_m , maximal fluorescence yield of light-adapted leaf; F_v/F_m , maximal PSII quantum yield; PSI, photosystems I; MS, mass spectrometry; MW, molecular weight; NBT, nitroblue tetrazolium; OTM, olive tail moment; PAM, pulse amplitude modulation; PFD, photosynthetic flux density; PSPA, purple sweet potato anthocyanins; PSII, photosystems II; ϕ_{PSII} , effective PSII quantum yield; qP , photochemical quenching; ROS, reactive oxygen species; SOD, superoxide dismutase; SPSS, statistical package for social sciences; UV, ultraviolet; VIS, visible; WT, wild-type.

The enzymatic system includes catalase, ascorbate peroxidase, superoxide dismutase (SOD), etc., while the non-enzymatic system includes ascorbic acid (AsA), α -tocopherol, glutathione, β -carotene, etc., (Allen et al. 2000, Foyer et al. 1994). In addition, along with other flavonoids, anthocyanins are also antioxidants that can scavenge ROS produced in plants (Duthie and Crozier 2000, Gould et al. 2002).

Anthocyanins are a group of flavonoid compounds responsible for the colors of many flowers, vegetables, fruits and berries (Aro et al. 1993, Chalker-Scott 1999). Synthesized from flavonoids via the shikimic acid pathway in the cytoplasm, they are transported into the vacuole by a glutathione pump (Marrs et al. 1995). Enhanced synthesis of anthocyanins in plants can be observed frequently when plants are exposed to physiological stresses such as high light, extreme temperature, (ultraviolet) UV radiation and drought or nutritional deficiency (Chalker-Scott 1999). The antioxidative capacity of purified anthocyanins has been demonstrated in many species in vitro (Garcia-Alonso et al. 2005, Tsai et al. 2002, Tsuda et al. 1996, Yamasaki et al. 1996). In particular, using the automated oxygen radical absorbance capacity to determine the antioxidant capacity of 14 anthocyanins, Wang et al. (1997) found that the capacities of cyanidin-3-glucoside to scavenge oxygen radicals was 3.5 times stronger than α -tocopherol. Some in vivo experiments have suggested that anthocyanins may act both as a sunscreen because they strongly absorb green-blue light, and as a scavenger of ROS (Gould et al. 1995, Hughes et al. 2005, Neill and Gould 2003, Smillie and Hetherington 1999, Steyn et al. 2002). However, Lee et al. (2003), in a survey of senescing leaves from 89 woody species, found no evidence of photoprotection by anthocyanins, based on predawn measurements of photosystems II (PSII) photochemical efficiency. Manetas et al. (2003) came to the same conclusion as Lee et al. (2003) for young red leaves of the Mediterranean tree *Quercus coccifera*. In short, there is still no agreement on whether anthocyanins act as scavengers of ROS in leaves. Therefore, additional direct or indirect evidence is needed to elucidate the physiological functions of anthocyanins.

In the present study, anthocyanins were isolated and purified from the root of purple sweet potato (*Ipomoea batatas* Lam.), which is known to be abundant in anthocyanins and to exhibit a strong antioxidative capacity (Oki et al. 2002, Tian et al. 2005). We used electron spin resonance (ESR) spectroscopy to monitor the free radical-scavenging ability of these anthocyanins in vitro. For investigating the physiological function of anthocyanins in vivo, the isolated anthocyanins of purple sweet potato (PSPA) were infiltrated into leaves

of *Arabidopsis* anthocyanin-deficient mutant (*tt3tt4*) and wild-type (WT). Our results suggest that anthocyanins in vitro and in vivo can alleviate the direct or indirect oxidative damage to the photosynthetic apparatus and DNA in plants caused by high-light stress.

Materials and methods

Extraction and purification of anthocyanins

Anthocyanins extraction was carried out as described by Longo et al. (2007). Purple sweet potato was extracted with 200 ml HCl : methanol solution (0.1:99.9, v/v) overnight in the dark. The extract was centrifuged at 5000 g for 30 min, and concentrated using a rotary evaporator at 35°C. The residue was dissolved in 0.01% (v/v) HCl in water. This aqueous solution was absorbed onto an activated C₁₈ Sep-Pak cartridge (Waters Corporation, Milford, MA). After washing with two volumes of 0.01% aqueous HCl and two volumes of ethyl acetate, anthocyanins were eluted with methanol containing 0.01% HCl (v/v). The acidified methanol solutions obtained were evaporated using a rotary evaporator at 30°C. The remaining solids were dissolved in 0.01% aqueous HCl and dried under reduced pressure. The powder was stored at –20°C until further analysis.

Analysis of the composition of anthocyanins

Chromatographic analysis of anthocyanins was performed on an Agilent 1100 Series LC/MSD-Trap-XCT system equipped with an electrospray ionization interface (ESI) and a diode array detector. A 4.6 × 250 mm, 5 μ m, Zorbax SB-C18 column (Agilent Technologies, Rising Sun, MD) was used for separation of anthocyanins. The mobile phase was composed of 1.5% formic acid aqueous solution (solvent A) and methanol (solvent B) at the flow rate of 0.8 ml min^{–1} with 20 μ l injection at 30°C, and detected at 525 nm. The elution gradient was performed as follows: 0–5 min, 10% B; 5–60 min, 60% B; 60–70 min, 60% B. Positive ion was applied with the following conditions: capillary voltage, 3.5 kV; dry temperature, 350°C; dry gas, 8.0 l min^{–1}; Nebulizer, 240 kPa; scan range 50–1500 m/z. Anthocyanins extracted from purple sweet potato were identified by mass spectrometry (MS) and UV–visible (VIS) data in comparison with reported literatures (Tian et al. 2005, Wu and Prior 2005).

Total anthocyanin contents in the powder obtained from purple sweet potato were determined by using a differential pH method (Wang and Lin 2000). A UV–VIS spectrophotometer UV2450 (Shimadzu Corp., Kyoto, Japan) and a 1-cm pathlength cell were used

for spectral measurements at 528 and 700 nm. Pigment content was calculated as cyanidin-3-glucoside; the molecular weight (MW = 449.2) and molar absorptivity ($\epsilon = 26900$) for cyanidin-3-glucoside were used. The resultant value was then expressed in term of weight of anthocyanins per dry weight of powder.

Antioxidant capacity in vitro: ESR assay

ESR spectra were obtained with a Bruker EMX-8 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) set at the X-band (25°C). AsA, SOD, and mannitol were each used as a reference scavenger of organic free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•, Sigma-Aldrich, St. Louis, Missouri, USA) superoxide radical and hydroxyl radical, respectively.

DPPH• scavenging activity

A methanol solution (60 μ l) of each sample (or ethanol itself as control) was added to 60 μ l of DPPH• (60 μ M; Sigma-Aldrich, St. Louis, Missouri, USA) in ethanol solution. After mixing vigorously for 10 s, the solutions were then transferred into a 100 μ l hematocrit capillary tube and fitted into the cavity of the ESR spectrometer. The spin adduct was measured exactly 2 min later. Measurement conditions were: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 5 mW; gain 6.3×10^5 ; temperature, 298 K. Different concentrations of pure cyanidin chloride (Cy, Sigma-Aldrich, St. Louis, Missouri, USA) and AsA were used for comparing the scavenging activity to DPPH• with PSPA.

Superoxide radical ($O_2^{\bullet-}$) scavenging activity

Superoxide radical-scavenging activity was measured as described by Peng et al. (2006) with minor modifications. The superoxide radical was produced by irradiating riboflavin. Riboflavin (0.1 mM, in 50 mM of phosphate buffer, pH 7.0) was mixed with 5 mM ethylenediaminetetraacetic acid, and the test anthocyanin sample (PSPA or Cy) in the presence or absence of a superoxide radical spin trap, Tiron (20 mM; 1,2-dihydroxybenzene-3,5-disulfonate, Sigma-Aldrich, St. Louis, Missouri, USA). The mixture was inserted into a hematocrit capillary tube and irradiated for 1 min with an UV light. ESR measurements were performed under the following conditions: modulation frequency, 100.00 kHz; modulation amplitude, 1.00 G; receiver gain, 2.52×10^4 ; time constant, 163.840 ms; conversion time 40.960 ms; center field, 3511.000 G; sweep width, 200.00 G; microwave power, 1.006 mW.

Hydroxyl radical ($\bullet OH$) scavenging activity

The hydroxy radical was generated by the Fenton reaction which was triggered by $FeSO_4$. After incubation for 2 min, 50 μ l of the mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 , 10 mM $FeSO_4$, 50 mM 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO, Sigma-Aldrich, St. Louis, Missouri, USA) and 50 μ l of a test compound (PSPA, Cy or mannitol) were quickly transferred to the hematocrit capillary tube. The ESR spectrometer settings were: microwave power, 2.007 mW; microwave frequency, 9.856 GHz; modulation amplitude, 0.1 mT; time constant, 164 ms; sweep time, 41.934 s; center field, 3511 G.

Plant growth conditions

Seeds of WT *Arabidopsis* ecotype Landsberg erecta (Ler) and Landsberg erecta mutant (*tt3tt4*), which was deficient in the dihydroflavonol 4-reductase locus and chalcone synthase locus, were germinated and grown in a phytotron at 22°C and a photoperiod of 8 h under photosynthetic flux density (PFD) of 100 μ mol $m^{-2} s^{-1}$ (Shao et al. 2008).

Pretreatment of leaves

Whole leaves of 4-weeks-old *Arabidopsis* were collected and vacuum infiltrated with a PSPA solution (0.04 mg ml^{-1}) or purified water, followed by exposure to high light (high voltage sodium lamp, 1300 μ mol $m^{-2} s^{-1}$) for 2 h. The temperature of the leaf surface was controlled at approximate 25°C by a circulating water bath. In addition, the absorption spectra of these leaves were recorded between 400 and 700 nm using a UV–VIS spectrophotometer.

Spectral analysis of *Arabidopsis* leaves

Anthocyanin was extracted in 3 ml of 1% (v/v) HCl in methanol, and 1.0 ml of distilled H_2O was added. The extracts were kept at 4°C overnight before chloroform extraction. After centrifugation for 2 min at 3000 g, the supernatant were recorded from 400 to 700 nm (Wade et al. 2003).

H_2O_2 and $O_2^{\bullet-}$ localization in situ

The method of ROS localization was conducted according to Romero-Puertas et al. (2004). For H_2O_2 in situ localization, H_2O_2 was visualized by diaminobenzidine (DAB) staining. Excised leaves from plants of WT or *tt3tt4*, continually irradiated under high light (1300 μ mol $m^{-2} s^{-1}$) for certain times (90 min, 180 min),

were vacuum infiltrated with a solution of DAB (0.5 mg ml^{-1} , Sigma-Aldrich, St. Louis, Missouri, USA) in 50 mM phosphate buffer (pH 7.0) for 10 min, before incubating at room temperature for 8 h in the absence of light. Then the leaves were illuminated with white light ($80 \mu\text{mol m}^{-2} \text{ s}^{-1}$) until the appearance of brown spots, characteristic of the reaction product of DAB with H_2O_2 . Chlorophyll was then bleached by immersion in boiling ethanol (75%, v/v) to visualize the brown spots, and leaves were photographed by a digital camera. For $\text{O}_2^{\bullet-}$ in situ localization, leaves were exposed to the high light, followed by vacuum-infiltrating for 5–10 min with a solution containing 50 mM K-phosphate buffer (pH 6.4), 10 mM Na-azide, and 0.1% nitroblue tetrazolium (NBT). Then the leaves were illuminated with white light ($80 \mu\text{mol m}^{-2} \text{ s}^{-1}$) until appearance of dark spots, characteristic of blue formazan precipitates. The subsequent steps were the same as for localization of H_2O_2 .

Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurement of leaves was conducted by an imaging pulse amplitude modulated chlorophyll fluorometer (IMAGING-PAM, Heinz Walz GmbH, Effeltrich, Germany) as described by Shao et al. (2008). Fluorescence was measured with relatively weak measuring light pulses ($<1 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at a low frequency (1 Hz) for measurement of minimum fluorescence yield of a dark-adapted leaf (F_0). Maximal fluorescence yield of dark-adapted leaf (F_m) was measured during an 800-ms exposure to a PFD of approximate $2700 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The intensity of continuous actinic illumination was adjusted to $185 \mu\text{mol m}^{-2} \text{ s}^{-1}$. All lighting (modulated measuring light, actinic light and saturation pulses for measurement of F_m and maximal fluorescence yield of light-adapted leaf, F'_m) was provided from blue light-emitting diodes (450 nm). Fluorescence measurements were started after 10–15 min dark adaptation. Values of the chlorophyll fluorescence parameters F_0 , F_m and F'_m , F_v/F_m (maximal PS II quantum yield), ϕ_{PSII} (effective PSII quantum yield) and photochemical quenching (qP) were each averaged over the area of interest ($n = 3$). In addition, their images were simultaneously derived from the IMAGING-PAM software.

Comet assay

The comet assay was performed according to Jiang et al. (2007) with minor modifications. Briefly, *Arabidopsis* leaves, similarly treated as above, were chopped in Galbraith buffer (45 mM MgCl_2 , 30 mM sodium citrate, 20 mM 4-morpholinepropanesulfonate and 0.01% Triton X-100). The nuclei were filtered through

$30\text{-}\mu\text{m}$ nylon mesh, and mixed with low melting point agarose. The mixture was then added onto the normal melting point agarose-treated microscope slide. After lyses, the slides were incubated in electrophoresis buffer for 25 min, followed by electrophoresis at 0.72 V cm^{-1} (19 V ; 300 mA) for 25 min at 4°C . After electrophoresis, the slides were air-dried, stained with SYBR Gold (1:10 000; Molecular Probe, Carlsbad, CA) and observed using a fluorescence microscope (Zeiss Axio100, Jena, Germany). Comet parameters were measured and analyzed using CASP software. The 'Olive Tail Moment' (OTM) [(tail profile center of gravity - head profile center of gravity) $\times$ tail % DNA] was calculated as a measure of DNA damage.

Statistical analysis

The statistical package for social sciences (SPSS Inc. 1998) was used for statistical analysis. For comparing results of different treatments, the Tukey analysis of variance test was performed. Any statistical probability ≤ 0.05 was considered significant.

Results

Characterization and total content of PSPA

In the present study, anthocyanins in the roots of purple sweet potato were found to consist of 10 components, identified as members of two classes of anthocyanidin aglycons (cyanidin and peonidin) based on the MW and product ion fragments using LC/MS (Table 1). The MWs of these 10 anthocyanins were in the range of 773–1111. As compared with a previous report (Tian et al. 2005), fewer anthocyanin components were detected in our study. The total content of anthocyanins in extracted powder of PSPA was $66.4 \pm 1 \text{ mg}$ per gram powder ($n = 3$) using cyanidin-3-glucoside as standard.

Antioxidant activities determined by ESR

The ESR technique is the most direct method to detect highly reactive, short-lived free radicals. In the present study, IC_{50} , representing the concentration necessary for causing a 50% inhibition of the ESR peak height, was used to compare the ability of Cy, PSPA and common or specific antioxidants to scavenge three different free radicals. Fig. 1 shows the ESR spectra of the DPPH $\bullet$ radical, superoxide radical and hydroxyl radical. Table 2 shows IC_{50} of PSPA and two other spin traps (Cy and AsA) in their inhibition of ESR signals of three free radicals. Scavenging the free radical DPPH $\bullet$ is a rapid and sensitive assay to evaluate the antioxidative

Table 1. MS data of anthocyanin compositions in purple sweet potato.

Anthocyanin	Retain-time (min)	MW	Fragment ions (m/z)
Cyanidin 3-sophoroside-5-glucoside	20.8	773	611 449 287
Peonidin 3-sophoroside-5-glucoside	24.6	787	625 463 301
Peonidin 3-caffeoylsophoroside-5-glucoside	25.7	949	787 463 301
Cyanidin 3-p-hydroxybenzoylsophoroside-5-glucoside	30.6	893	731 449 287
Peonidin 3-p-hydroxybenzoylsophoroside-5-glucoside	34.5	907	745 463 301
Cyanidin 3-(6''-feruloylsophoroside)-5-glucoside	36.6	949	787 449 287
Cyanidin 3-(6''-caffeoyl-6''-feruloylsophoroside)-5-glucoside	40	1111	949 449 287
Cyanidin 3-caffeoylsophoroside-5-glucoside	40.1	935	773 449 287
Peonidin 3-(6''-feruloylsophoroside)-5-glucoside	40.4	963	801 663 301
Peonidin 3-caffeoyl-p-hydroxybenzoyl-sophoroside-5-glucoside	44.6	1069	907 463 301

capability of natural antioxidants. The ESR spectrum of the DPPH• free radical revealed a five-peak pattern, which was consistent with the observation of Ahn et al. (2007). PSPA, AsA and Cy all could significantly reduce the peak height of the DPPH• spectrum (Fig. 1). The IC₅₀ value of PSPA was greater than that of Cy but one-fold less than that of AsA, indicating that PSPA is an effective scavenger of DPPH• (Table 2).

The superoxide radical was generated by irradiating riboflavin and trapped by Tiron. The spectrum of the superoxide radical in the presence of PSPA, Cy, SOD or their absence as control are shown in Fig. 1. PSPA and Cy exhibited an obvious superoxide radical-scavenging activity. Making a comparison between their IC₅₀ values, it was found that the superoxide radical-scavenging activities decreased in the order of PSPA (14.8 µM) > SOD (39.1 µM) > Cy (58.6 µM).

DMPO is a trap for the hydroxyl radical which is produced by the Fenton reaction. The typical four-line

ESR spectrum of DMPO-OH adduct was consistent with that reported by Peng et al. (2006) and Ahn et al. (2007) (Fig. 1). Mannitol, a potential scavenger of •OH, was observed with very low hydroxyl radical-scavenging activity, the IC₅₀ being 4530 µM. This result is in accordance with the observations by Peng et al. (2006). However, the IC₅₀ of Cy and PSPA was 52 and 85 µM, respectively, reflecting the strong capacity of anthocyanins for scavenging hydroxyl radicals.

Response of chlorophyll fluorescence of leaves of *Arabidopsis tt3tt4* to high-light treatment after infiltration with PSPA

To investigate whether some PSPA had indeed been infiltrated into the leaves, we extracted the anthocyanins from the leaves with acid methanol, and measured the absorbance spectrum in vitro (Fig. 2). A higher absorbance peak at around 530 nm was observed, albeit on an expanded scale, in extraction from double mutant leaves infiltrated with PSPA (0.04 mg ml⁻¹) than in the non-infiltrated WT leaves. By contrast, in the anthocyanin-deficient mutant *tt3tt4*, no absorption peak was detected at 530 nm, indicating that anthocyanin biosynthesis was disrupted in the double mutant. These results show that some PSPA had indeed been infiltrated into the *tt3tt4* leaves.

Fig. 1. ESR spectra of spin adducts of DPPH• radical, superoxide radical (trapped by Tiron, 20 mM) and hydroxyl radical (trapped by DMPO, 50 mM).

Table 2. IC₅₀ of scavenging three radicals determined by ESR.

Radicals	Antioxidants	IC ₅₀ (µM)
DPPH•	PSPA	7.4
	Cy	4.7
	AsA	15.6
Superoxide radical	PSPA	14.8
	Cy	58.6
	SOD	39.1
Hydroxyl radical	PSPA	85
	Cy	52
	D-mannitol	4530

Fig. 2. Absorbance spectra of anthocyanins extracted from WT and *tt3tt4* mutant leaves with acid methanol, as well as double mutant leaves preinfiltrated with 0.04 mg ml⁻¹ PSPA.

Chlorophyll fluorescence parameters are useful indices for the characterization of photosynthetic performance, particularly under stress conditions. F_v/F_m decreased significantly in *tt3tt4* leaves preexposed to high light (1300 $\mu\text{mol m}^{-2} \text{s}^{-1}$) as compared with leaves in the dark. However, in leaves preinfiltrated with PSPA (0.04 mg ml⁻¹) and exposed to the same high-light condition thereafter, the decreasing trend of F_v/F_m was ameliorated by 19% at 300 min (Fig. 3A). The effective PSII yield (ϕ_{PSII}) was also reduced by high-light treatment (Fig. 3B). A sudden drop of ϕ_{PSII} appeared after illumination for 30 min, but a significant difference between the presence and absence of PSPA was found beyond 180 min of illumination. After treatment of leaves for 300 min, ϕ_{PSII} of high-light treated leaves infiltrated with PSPA was almost double that of leaves infiltrated with water. Fig. 3C shows the changing dynamics in qP among the three treatments. qP showed a similar trend as ϕ_{PSII} , the significant changes in qP between the two high-light treatments occurring after exposure of leaves to high light for ≥ 180 min; the qP of PSPA-infiltrated leaves at 300 min of high-light treatment was ca. double of that of leaves infiltrated with water. In addition, as we can see, leaves kept in the dark had only a slight change in F_v/F_m , ϕ_{PSII} as well as qP during this time course (Fig. 3). The changes in chlorophyll fluorescence parameters of the leaves exposed to high light for 300 min were confirmed by the color-coded fluorescence images (Fig. 4). No significant differences between 'Dark + water' and 'Dark + PSPA' were detected among these parameters. For either F_v/F_m or ϕ_{PSII} , the image color of PSPA-infiltrated leaves was obviously intermediate between the dark control and the (HL + water)-treated leaves. These images show

Fig. 3. Effects of exogenous PSPA on chlorophyll fluorescence parameters of leaves of the anthocyanin-deficient mutant (*tt3tt4*) exposed to high light for up to 300 min. (A) F_v/F_m ; (B) ϕ_{PSII} ; (C) qP . Data are presented as means $\pm$ SD ($n \geq 5$).

Fig. 4. Changes in fluorescence images of F_v/F_m , ϕ_{PSII} and qP in four different treatments given to leaves of the mutant *tt3tt4*. Whole leaves were exposed to a solution of 0.5 mg ml⁻¹ PSPA for 300 min. Fluorescence images are indicated by the false color code at the bottom. The color code ranges from black via red, orange, yellow, green, blue and violet to purple, and these colors code for numbers between 0 and 1. 'Dark + water': leaves infiltrated with water in the dark; 'Dark + PSPA': leaves infiltrated with PSPA in the dark; 'HL + PSPA': leaves infiltrated with PSPA and exposed to the high light; 'HL + water': leaves infiltrated with water and exposed to the high light.

that additional anthocyanins in the leaves could alleviate photodamage to *tt3tt4* mutant to a certain extent.

Endogenous anthocyanins in WT *Arabidopsis* leaves play a protective role in high-light stress

High levels of ROS accumulate in the cell when plants are continuously exposed to light higher than that required to saturate photosynthesis. Here, hydrogen peroxide (H_2O_2) and superoxide radical were detected

Fig. 5. H_2O_2 (left panel) and superoxide radical (right panel) localization in situ in leaves of WT and *tt3tt4* mutant. *Arabidopsis* leaves were preirradiated with or without high light ($1300 \mu\text{mol m}^{-2} \text{s}^{-1}$) for time indicated, followed by infiltrating with DAB or NBT for visualizing H_2O_2 and superoxide radical, respectively.

in leaves of WT *Arabidopsis* and *tt3tt4* mutant with DAB and NBT staining, respectively (Fig. 5). In H_2O_2 detection, the brown deposits are the result of the reaction of DAB with H_2O_2 . Control leaves of two phenotypes (WT and *tt3tt4*) were stained by some pale brown deposits, without significant differences between them; these deposits may have been caused by the DAB itself; as Snrychová et al. (2009) found that DAB may have a toxic effect. By contrast, compared with the WT leaf, more brown deposits could be observed in the leaf of the mutant when both were exposed to the high light ($1300 \mu\text{mol m}^{-2} \text{s}^{-1}$) for even just 90 min. Furthermore, more obvious distinction appeared at 180 min illumination between the two phenotypes. In superoxide radical detection, where the blue formazan deposits were characteristic of reaction of NBT with superoxide radical, the result was very similar with the H_2O_2 detection. When exposed to the high light, a leaf of the *tt3tt4* mutant was stained more intensely than WT leaf, and obviously, the leaf accumulated more deposits when illuminated for 180 min than for 90 min in each phenotype. The results indicated that H_2O_2 and superoxide radicals were produced when excised leaves of *Arabidopsis* were exposed to high light, and anthocyanin-deficient mutant leaves may be more sensitive to high-light stress, which is consistent with the results of chlorophyll fluorescence measurements (Figs 3 and 4). In other words, we have demonstrated that endogenous anthocyanins in WT *Arabidopsis* leaves play a protective role in high-light stress. We next used the double mutant to show the protective effects of exogenous anthocyanins, PSPA.

Supplemental PSPA alleviated DNA damage in *Arabidopsis* leaves induced by high light

ROS cause the breakdown of double-stranded DNA in the cell (Beckman and Ames 1997, Shulaev and

Fig. 6. Supplemental PSPA (0.5 mg ml^{-1}) alleviated DNA damage induced by high light for 300 min in leaves of *Arabidopsis*. (A) comet assay images of *Arabidopsis* leaves exposed to high light ($1300 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) DNA damage calculated by OTM from comet assay images. The first line in (A), and the black bar in (B) represent WT; the second line in (A), and the gray bar in (B) represent the *tt3tt4* mutant. 'Dark + water': leaves infiltrated with water in the dark; 'HL + water': leaves infiltrated with water exposed to the high light; 'HL + PSPA': leaves infiltrated with PSPA exposed to the high light; 'HL + Cy': leaves infiltrated with $40 \mu\text{mol}$ Cy exposed to the high light. Data shown are means ($\pm \text{SD}$) of three independent replicates.

Oliver 2006). In the present study, we compared the susceptibility of WT and *tt3tt4* *Arabidopsis* leaves to ROS-induced damage. Damage of DNA was determined using the comet assay, which has recently been applied to measure DNA damage in plants (Jiang et al. 2007). In darkness, a short comet tail was observed in the WT or *tt3tt4*. By contrast, after leaves had been exposed to high light ($1300 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 300 min, the extent of the tail enlarged markedly. In the WT, tail enlargement was less than that of *tt3tt4*, implying that the DNA damage in WT was less than in *tt3tt4*. However, when leaves were infiltrated with PSPA or Cy instead of water before illumination with high light, the tails became much shorter. Irrespective of supplementation with PSPA or Cy, DNA tails in WT were always shorter than those in *tt3tt4* (Fig. 6A).

The effect of supplemental anthocyanins on the OTM, determined from fluorescence images in the leaves of *Arabidopsis* phenotype, is shown in Fig. 6B. In the dark, no significant difference was found in the extent of OTM

between WT and *tt3tt4*, both infiltrated with water. After high-light exposure, however, OTM increased markedly in both WT and *tt3tt4*, though OTM in WT was 37% less than in *tt3tt4*. The increase of OTM caused by high light was alleviated to a considerable extent when anthocyanins were infiltrated into the leaves before leaves were subjected to such stress.

Discussion

Purple sweet potato anthocyanins and its antioxidative capability in vitro

Anthocyanins are glycosides and acylglycosides of anthocyanidins. Based on their different hydroxyl or methoxyl substitutions in their basic structure, approximately 400 anthocyanins existed in nature. The most common anthocyanidin aglycons in plants are cyanidin, delphinidin, malvidin, petunidin, peonidin and pelargonidin (Tian et al. 2005, Wang et al. 1997). Purple sweet potato (*I. batatas* Lam.) has been found to be abundant in anthocyanins. It was reported that the purple sweet potato cultivar 'Ayamurasaki' contained higher content of anthocyanins than in other cultivars (Konczak-Islam et al. 2003). A total of 26 anthocyanins were detected and characterized in the cultivar 'Ayamurasaki' by high-performance liquid chromatography–ESI–MS/MS, including two pelargonidin derivatives (Tian et al. 2005). By using the pH differential method as described by Wang and Lin (2000), anthocyanins made up 6.6% (w/w) of our PSPA powder. Using the MWs and MS fragments of anthocyanins detected by Tian et al. (2005) as reference, at least 10 anthocyanins were found in our purified sample of purple sweet potato. All these anthocyanins were cyanidin 3-sophoroside-5-glucoside or peonidin 3-sophoroside-5-glucoside, or their derivatives acylated by ferulic acid or caffeic acid (Table 1). Fewer anthocyanin components were detected in our study perhaps because of the differences in growth conditions, analysis technique and cultivar. Further, to determine the anthocyanins qualitatively and quantitatively is very difficult because of their complicated and similar basic structures, and to a paucity of authentic anthocyanin standards.

Strong potential antioxidative capability of anthocyanins has been extensively demonstrated and confirmed in in vitro assays (Garcia-Alonso et al. 2005, Tsai et al. 2002, Tsuda et al. 1996, Yamasaki et al. 1996). It has been suggested that different antioxidative capacities arise from the different position and amount of hydroxyl groups in the aromatic ring of the anthocyanin structure, which can donate an electron to a free radical (Garcia-Alonso et al. 2005). Anthocyanins extracted from purple

sweet potato have been found to have a strong antioxidative capability (Oki et al. 2002). The present study using the ESR technique provides direct evidence of significant capability of PSPA to scavenge ROS and organic free radicals, including superoxide radical, hydroxyl radical and stable DPPH•. According to the IC₅₀ values in Table 2, PSPA was more potent than AsA against DPPH•, more potent than SOD against O₂•, and more potent than mannitol against •OH, possibly because of the function of multiple components in our PSPA. Further, it is seen that PSPA and Cy revealed a stronger radical-scavenging capability against DPPH• than against O₂• and •OH. Because ROS are short-lived species (Shulaev and Oliver 2006), we speculate that one main reason for the differential scavenging capability against different ROS may be because of the different lifetimes of free radicals. The activity and efficiency of spin trapping of ROS might be limited by their short lives, while DPPH• is a stable organic free radical, which is easily trapped.

Photoprotective in vivo effects of PSPA on *Arabidopsis* leaves under high light

ROS, including singlet oxygen, superoxide radical and hydrogen peroxide, are often generated as by-products of physiological reactions or induced by exogenous stress factors present in high-light conditions. These very transient species with high chemical reactivity may lead to damage of proteins, DNA, as well as lipids (Fryer et al. 1998, Mancini et al. 2006). Fig. 5 visualized the production of superoxide radical and hydrogen peroxide under high-light exposing leaves of WT and *tt3tt4* mutant. Using digital imaging to identify the sites of photooxidative stress in *Arabidopsis* leaves, Fryer et al. (2002) found that excess light led to an increase in the generation of single oxygen, superoxide radical and hydrogen peroxide.

Anthocyanins have been suggested to possess the potential to alleviate photooxidation, as a screen to shield chloroplasts from excess absorbed light energy and/or as an antioxidant that scavenges ROS (Gould et al. 2002, Neill and Gould 2003, Shao et al. 2008). Pietrini and Massacci (1998) found that approximately 43% incident light can be absorbed by anthocyanins in the epidermal vacuoles of *Zea mays* L. leaves. Recently, our previous study (Shao et al. 2008) demonstrated that WT of *Arabidopsis* tolerated photooxidative damage induced by methyl viologen better than *tt3tt4*. Otherwise, there was no significant difference in absorption spectra between the leaves of WT and *tt3tt4* mutant, which indicated that anthocyanin with low concentration in WT of *Arabidopsis* had little effect on the light energy absorption. Our result of supplemental PSPA

in *Arabidopsis* leaves also showed no contribution to leaf absorption spectra (data not shown). However, higher absorption peak at 530 nm was detected in PSPA-infiltrated *tt3tt4* leaves than WT leaves (Fig. 2). It illustrates that low concentration PSPA was indeed diffused into the leaves. In this paper, therefore, we could explore antioxidant capability of anthocyanin in vivo by avoiding its characteristic of intensively absorbing excessive light energy.

A fluorescence imaging system with three parameters (F_v/F_m , ϕ_{PSII} and qP) was used to analyze the effect of supplemental PSPA on PSII function in *tt3tt4 Arabidopsis* leaves preexposed to high-light stress. Deficiency in the anthocyanin synthesis gene increased the susceptibility to high-light stress, resulting in much greater reduction in F_v/F_m , ϕ_{PSII} and qP in the *tt3tt4* mutant after 300 min of high-light exposure, but these reductions could be alleviated by exogenous PSPA (Fig. 3). The higher values of F_v/F_m , ϕ_{PSII} and qP in PSPA-treated leaves after 300 min high-light treatment lead us to conclude that anthocyanins in vivo or/and in vitro all enhance protection of photosynthetic apparatus in high light.

The time course of changes in the fluorescence parameters during high-light treatment is intriguing. At 30 min high-light treatment time, both ϕ_{PSII} and qP decreased abruptly, while F_v/F_m changed very little. This initial effect could have been because of photodamage to photosystems I (PSI) because of the production of ROS, initiated by the reduction of oxygen on the acceptor side of PSI. Inhibition of PSI would slow electron flow out of the plastoquinone pool, resulting in a more reduced state of the primary quinone acceptor in PSII, and therefore a lower qP . Because $\phi_{PSII} = qP \times F'_v/F'_m$, where F'_v/F'_m is the photochemical quantum yield of open PSII reaction center traps, ϕ_{PSII} and qP were expected to change in parallel, as observed (Fig. 3B, C).

Interestingly, no photoprotective effect was observed for the first 180 min treatment in the anthocyanin biosynthesis deficient mutant after infiltrated with PSPA (Fig. 3B, C). We suggested that chloroplast can promptly initiate its protecting systems when the photosynthetic apparatus was exposed to not so serious environmental stresses, by dissipating light energy as heat through Stern–Volmer non-photochemical quenching (which remained high during the first 120 min of illumination, data not shown), adjusting light-harvesting antenna size, synthesizing stress protein, accumulating lipophilic and water-soluble antioxidant molecules as well as enhancing scavenging enzymatic systems (Chalker-Scott 1999, Demmig-Adams and Adams 1992, Niyogi 1999). Our previous study also confirmed that anthocyanins exhibited no photoprotection at the beginning of photooxidation treatment (Shao et al. 2008). These can also explain

why some reports showed no photoprotection of anthocyanin against stresses (Lee et al. 2003, Manetas et al. 2003). However, as the duration of high-light illumination treatment increased, leaves were under severe stress, causing PSII itself to apparently suffer considerable photodamage, and numerous ROS such as $O_2^{\bullet-}$ and H_2O_2 could be induced (Fig. 5). The photoprotection systems in chloroplast were not enough to eliminate numerous ROS. This resulted in accumulation of ROS in chloroplast. $O_2^{\bullet-}$ in aqueous solution was rapidly protonated to form the $\bullet OH$, or else was converted by SOD to H_2O_2 , both of which can freely permeate the whole cell (Takahashi and Asada 1983, Yamasaki et al. 1997). In Fig. 5, less H_2O_2 and $O_2^{\bullet-}$ accumulation were observed in the leaves of WT than those of *tt3tt4* mutant, demonstrated that anthocyanin can reduce ROS accumulation. PSPA exert its strong ROS scavenging capability (Table 2 and Fig. 1) to protect oxidative damage, which can explain that PSPA-infiltrated leaves had higher ϕ_{PSII} and qP than that without PSPA after 180 min light treatment (Fig. 3B, C). Therefore, PSPA may compensate for photoprotection through scavenging excessive ROS under severe stress.

DNA is one of the major targets of ROS. ROS can cause breakdown or modification of DNA strands. In the present study, the pronounced alteration of OTM parameters in leaves of *Arabidopsis* WT and *tt3tt4* under high-light treatment indicated that DNA was attacked by ROS induced by high light, followed by breaks in the double strands. This kind of oxidative DNA damage occurred in the same manner as in animal cells (Kumaravel and Jha 2006), but is seldom reported in plants exposed to high light. The lower OTM values in *Arabidopsis* leaves infiltrated with PSPA or Cy corroborated the protective role of anthocyanins against DNA damage in eliminating the attack by free radicals. Interestingly, the effect of the *endogenous* anthocyanins in the WT can be quantified by the OTM difference between WT and *tt3tt4* (Fig. 6B) in the (HL + water) treatment (25.0 units) or the (HL + PSPA) treatment (24.1 units), these two values being similar. Similarly, the effect of *exogenous* PSPA is given by the difference between the (HL + water) treatment and the (HL + PSPA) treatment in the WT (48.9 units) or in *tt3tt4* (49.6 units), these two values being similar. Finally, the effect of exogenous Cy is given by the difference between the (HL + water) treatment and the (HL + Cy) treatment in the WT (55.9 units) or in *tt3tt4* (58.9 units), these two values being similar. The similarity within each pair of values for WT or the *tt3tt4* mutant indicates that each set of exogenous anthocyanins (PSPA or Cy) protected the DNA equally well in the WT and the *tt3tt4* mutant. The result that exogenous anthocyanins mitigated the DNA damage may have come about

because light-induced ROS, which can enter the nucleolus to attack DNA, were eliminated by anthocyanins.

In conclusion, 66 mg anthocyanins g⁻¹ of powder was detected in purple sweet potato tubers and identified to consist of 10 components. The isolated PSPA had a strong capability for scavenging ROS radicals O₂^{-•} and •OH, as well as DPPH•. Supplemental PSPA in leaves of *Arabidopsis* anthocyanin-deficient mutant *tt3tt4* alleviated DNA damage and photoinhibition of PSII under strong light stress. Our results provide direct evidence for the role of PSPA in protection against free radicals and their mediated damage to DNA and the photosynthetic apparatus.

Acknowledgements – We thank professor Zhi-Fang Lin (South China Botanical Garden, Chinese Academy of Sciences, China) for critical reading and helpful suggestions. Furthermore, we are grateful to Qun Gu (Zeal Quest Scientific Technology Co. Ltd., China) and Lei Jiang (College of Life Science, South China Normal University, China) for their equipments and technical assistances. This work was supported by National Natural Science Foundation of China (30770173, 30870385) and National Basic Research Program of China (973 Program) (2009CB118504).

References

- Ahn GN, Kim KN, Cha SH, Song CB, Lee J, Heo MS, Yeo IK, Lee NH, Jee YH, Kim JS, Heu MS, Jeon YJ (2007) Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H₂O₂-mediated DNA damage. *Eur Food Res Technol* 226: 71–79
- Allen J, Asada K, Foyer CH, Matthijs HCP (2000) The water-water cycle as alternative photon and electron sinks – discussion. *Phil Trans R Soc Lond B Biol Sci* 355: 1430–1431
- Aro E-M, Virgin I, Andersson B (1993) Photoinhibition of photosystem II: Inactivation, protein damage and turnover. *Biochim Biophys Acta* 1143: 113–134
- Beckman KB, Ames BN (1997) Oxidative decay of DNA. *J Biol Chem* 272: 19633–19636
- Chalker-Scott L (1999) Environmental significance of anthocyanins in plant stress responses. *Photochem Photobiol* 70: 1–9
- Demmig-Adams B, Adams WW (1992) Photoprotection and other responses of plants to high light stress. *Annu Rev Plant Physiol Plant Mol Biol* 43: 599–626
- Duthie G, Crozier A (2000) Plant-derived phenolic antioxidants. *Curr Opin Lipidol* 11: 43–47
- Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. *Physiol Plant* 92: 696–717
- Fryer MJ, Andrews JR, Oxborough K, Blowers DA, Baker NR (1998) Relationship between CO₂ assimilation, photosynthetic electron transport, and active O₂ metabolism in leaves of maize in the field during periods of low temperature. *Plant Physiol* 116: 571–580
- Fryer MJ, Oxborough K, Mullineaux PM, Baker NR (2002) Imaging of photo-oxidative stress responses in leaves. *J Exp Bot* 53: 1249–1254
- Garcia-Alonso M, Rimbach G, Sasai M, Nakahara M, Matsugo S, Uchida Y, Rivas-Gonzalo JC, De Pascual-Teresa S (2005) Electron spin resonance spectroscopy studies on the free radical scavenging activity of wine anthocyanins and pyranoanthocyanins. *Mol Nutr Food Res* 49: 1112–1119
- Gould KS, Kuhn DN, Lee DW, Oberbauer SF (1995) Why leaves are sometimes red. *Nature* 378: 241–242
- Gould KS, McKelvie J, Markham KR (2002) Do anthocyanins function as antioxidants in leaves? Imaging of H₂O₂ in red and green leaves after mechanical injury. *Plant Cell Environ* 25: 1261–1269
- Hughes NM, Neufeld HS, Burkey KO (2005) Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. *New Phytol* 168: 575–587
- Jiang L, Wang Y, Li SS (2007) Application of the comet assay to measure DNA damage induced by UV radiation in the hydrophyte, *Spirodela polyrrhiza*. *Physiol Plant* 129: 652–657
- Konczak-Islam I, Okuno S, Yoshimoto M, Yamakawa O (2003) Composition of phenolics and anthocyanins in a sweet potato cell suspension culture. *Biochem Eng J* 14: 155–161
- Kumaravel TS, Jha AN (2006) Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat Res Gen Toxicol Environ Mutagen* 605: 7–16
- Lee DW, O'Keefe J, Holbrook NM, Feild TS (2003) Pigment dynamics and autumn leaf senescence in a New England deciduous forest, eastern USA. *Ecol Res* 18: 677–694
- Longo L, Scardino A, Vasapollo G (2007) Identification and quantification of anthocyanins in the berries of *Pistacia lentiscus* L., *Phillyrea latifolia* L. and *Rubia perigrina* L. *Innov Food Sci Emerg Technol* 8: 360–364
- Mancini A, Buschini A, Restivo FM, Rossi C, Poli P (2006) Oxidative stress as DNA damage in different transgenic tobacco plants. *Plant Sci* 170: 845–852
- Manetas Y, Petropoulou Y, Psaras GK, Drinia A (2003) Exposed red (anthocyanic) leaves of *Quercus coccifera* display shade characteristics. *Funct Plant Biol* 30: 265–270
- Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione-S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2. *Nature* 375: 397–400

- Neill SO, Gould KS (2003) Anthocyanins in leaves: light attenuators or antioxidants? *Funct Plant Biol* 30: 865–873
- Niyogi KK (1999) Photoprotection revisited: genetic and molecular approaches. *Annu Rev Plant Physiol Plant Mol Biol* 50: 333–359
- Oki T, Masuda M, Furuta S, Nishiba Y, Terahara N, Suda I (2002) Involvement of anthocyanins and other phenolic compounds in radical-scavenging activity of purple-fleshed sweet potato cultivars. *J Food Sci* 67: 1752–1756
- Peng CL, Lin ZF, Su YZ, Lin GZ, Dou HY, Zhao CX (2006) The antioxidative function of lutein: electron spin resonance studies and chemical detection. *Funct Plant Biol* 33: 839–846
- Pietrini F, Massacci A (1998) Leaf anthocyanin content changes in *Zea mays* L. grown at low temperature: significance for the relationship between the quantum yield of PSII and the apparent quantum yield of CO₂ assimilation. *Photosynth Res* 58: 213–219
- Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. *Annu Rev Plant Physiol* 35: 15–44
- Romero-Puertas MC, Rodriguez-Serrano M, Corpas FJ, Gomez M, Del Rio LA, Sandalio LM (2004) Cadmium-induced subcellular accumulation of O₂^{•−} and H₂O₂ in pea leaves. *Plant Cell Environ* 27: 1122–1134
- Shao L, Shu Z, Peng CL, Lin ZF, Yang CW, Gu Q (2008) Enhanced sensitivity of *Arabidopsis* anthocyanin mutants to photooxidation: a study with fluorescence imaging. *Funct Plant Biol* 35: 714–724
- Shulaev V, Oliver DJ (2006) Metabolic and proteomic markers for oxidative stress. New tools for reactive oxygen species research. *Plant Physiol* 141: 367–372
- Smillie RM, Hetherington SE (1999) Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica* 36: 451–463
- Snyrchová I, Ayaydin F, Hideg E (2009) Detecting hydrogen peroxide in leaves in vivo – a comparison of methods. *Physiol Plant* 135: 1–18
- Steyn WJ, Wand SJE, Holcroft DM, Jacobs G (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytol* 155: 349–361
- Takahashi MA, Asada K (1983) Superoxide anion permeability of phospholipid-membranes and chloroplast thylakoids. *Arch Biochem Biophys* 226: 558–566
- Tian QG, Konczak I, Schwartz SJ (2005) Probing anthocyanin profiles in purple sweet potato cell line (*Ipomoea batatas* L. cv. Ayamurasaki) by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry. *J Agric Food Chem* 53: 6503–6509
- Tsai PJ, McIntosh J, Pearce P, Camden B, Jordan BR (2002) Anthocyanin and antioxidant capacity in Roselle (*Hibiscus sabdariffa* L.) extract. *Food Res Int* 35: 351–356
- Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T (1996) Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochem Pharmacol* 52: 1033–1039
- Wade HK, Sohal AK, Jenkins GI (2003) Arabidopsis ICX1 is a negative regulator of several pathways regulating flavonoid biosynthesis genes. *Plant Physiol* 131: 707–715
- Wang SY, Lin HS (2000) Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J Agric Food Chem* 48: 140–146
- Wang H, Cao GH, Prior RL (1997) Oxygen radical absorbing capacity of anthocyanins. *J Agric Food Chem* 45: 304–309
- Wu XL, Prior RL (2005) Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: Vegetables, nuts, and grains. *J Agric Food Chem* 53: 3101–3113
- Yamasaki H, Uefuji H, Sakihama Y (1996) Bleaching of the red anthocyanin induced by superoxide radical. *Arch Biochem Biophys* 332: 183–186
- Yamasaki H, Sakihama Y, Ikehara N (1997) Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H₂O₂. *Plant Physiol* 115: 1405–1412