The antioxidative role of anthocyanins in *Arabidopsis* under high-irradiance

Q. ZHANG[1], L.-J. SU[1], J.-W. CHEN[2], X.-Q. ZENG[1], B.-Y. SUN[1], and C.-L. PENG[1]*

College of Life Science, South China Normal University, Guangzhou 510631, Guangdong, P.R. China[1]
College of Agronomy and Biotechnology, Yunnan Agricultural University, Kunming 650201, Yunnan, P.R. China[2]

Abstract

To uncover the potential antioxidative role of anthocyanins *in vivo* in protecting photosynthetic tissues from photoinhibition, the effects of high irradiance [HI, 1300 μmol(photon) m^{-2} s^{-1}] were studied using detached leaves derived from *Arabidopsis* wild-type (WT) and the mutant deficient in anthocyanin biosynthesis (*tt3tt4*). HI stress caused decreased chlorophyll content and photochemical efficiency, but increased cell-membrane leakage and contents of hydrogen peroxide and superoxide radical in the leaves of both *Arabidopsis* phenotypes, but the WT plants showed better HI tolerance than *tt3tt4* mutant. HI caused a significant increase in the 1,1-diphenyl-2-picrylhydrazyl scavenging capacity in WT but not in the *tt3tt4* mutant. The anthocyanins could not contribute substantially to light-shielding during the periods of HI stress, because the anthocyanin content in WT was very low and the colour of leaves was the same as in the *tt3tt4* mutant. Thus, it was assumed that the better HI tolerance in WT was mostly related to the potential antioxidative role of anthocyanins.

Additional key words: chlorophyll fluorescence imaging, hydrogen peroxide, membrane leakage, mutant, superoxide radical.

Introduction

Anthocyanins are plant pigments produced by the flavonoid biosynthetic pathway. They are often present in flowers, fruits, leaves and stems, ranging in colour from orange/red to purple/blue. Anthocyanins function in flowers and fruits primarily to attract pollinators and seed distributors (Chalker-Scott 1999, Gould and Lister 2006).

Many environmental factors (*e.g.* light, temperature, nutrition, drought and infection) have an effect on the synthesis of anthocyanins (De Jong 1991, Beggs and Wellmann 1994, Meng *et al.* 2004, Piovan and Filippini 2007, Ismail and Mohamed 2010). In recent years, further research in the functional roles of anthocyanins became possible with new research methods (Agati *et al*. 2007).

Recently, the role of anthocyanins in photoprotection has been proposed (Gould *et al.* 1995, Field *et al.* 2001, Hoch *et al.* 2001). According to this hypothesis, the photoprotective roles of anthocyanins can be fulfilled in two ways: either by simply screening visible radiation or/and by radical scavenging (Wang *et al.* 1997, Archetti *et al.* 2009). The photoprotective roles, in which anthocyanins act as sunscreens to attenuate the visible radiation penetrating the mesophyll and thus reduce the excitation pressure, have been extensively reported (Smillie and Hetherington 1999, Field *et al.* 2001, Steyn *et al.* 2002, Manetas *et al.* 2003, Merzlyak *et al.* 2008). However, anthocyanins may also indirectly protect plants against excess radiation by their oxy-radical scavenging properties (Gould *et al.* 2002a, Steyn *et al.* 2002). Our previous study also indicated that leaves containing anthocyanins had a significantly greater antioxidant potential than did green leaves (Peng *et al.* 2006). Although some studies have confirmed that anthocyanins are powerful antioxidants (Tsuda *et al.* 1996, Wang *et al.* 1997, Gould *et al.* 2002a, Neill and Gould 2003), direct experimental evidence for the *in vivo* engagement of anthocyanins in antioxidative defense of leaves is lacking, and indirect indications are sparse (Manetas 2006).

Excess irradiance may be harmful for plants that are

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Abbreviations: $OH^{\bullet}$ - hydroxyl radical; CHS - chalcone synthase; DAB - diaminobenzidine; DFR - dihydroflavono-4-reductase; DPPH - 1,1-diphenyl-2-picrylhydrazyl; ETR - apparent electron transport rate; F_v/F_m - variable to maximum fluorescence ratio (the maximum photosystem 2 quantum yield); NBT - nitroblue tetrazolium; $O_2^{\bullet -}$ - superoxide radical; qP - the coefficient of photochemical quenching; ROS - reactive oxygen species; *tt3tt4* - *tt3tt4* mutant deficient in anthocyanin biosynthesis; WT - wild-type; Φ_{PS2} - effective photosystem 2 quantum yield.
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* Corresponding author: fax: (+86-20) 85212078, e-mail: pengchl@scib.ac.cn

unable to balance the absorbed/utilized energy ratio (Huner *et al.* 1998). When the absorption of light energy exceeds the capacity of photosynthesis and the photoprotective mechanisms are overwhelmed, reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$), can be produced (Havaux and Niyogi 1999), thereby resulting in oxidative stress. Hence, high irradiance can be used as a specific generator of ROS. In the present study, the wild-type and the anthocyanin-deficient mutant of *Arabidopsis* (*tt3tt4*) were used to investigate the antioxidative potential of anthocyanins. Our working hypothesis was that anthocyanins *in vivo* function to relieve stress caused by high irradiance through their powerful antioxidative capacity.

Materials and methods

Seeds of wild-type (WT) *Arabidopsis thaliana* ecotype Landsberg erecta (*Ler*) and the Landsberg erecta mutant deficient in anthocyanin biosynthesis (*tt3tt4*), which is deficient in both the dihydroflavonol 4-reductase (DFR) locus and the chalcone synthase (CHS) locus (Shirley *et al.* 1995, Peer *et al.* 2001), were obtained from the *Arabidopsis* Biological Resource Center, Columbus, OH, USA. The seed coat of the WT is brown, while the coat of the mutants is yellow. Seeds were imbibed for 2 d at 4 °C in the dark to synchronise germination, and then sown on sterilised compost. Plant seedlings were grown routinely in a growth cabinet at temperature of 20 - 22 °C, 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1} and relative humidity of 80 %. The third and fourth mature leaves from 25-d-old plants were used for the present study.

One set of leaves cut from the *tt3tt4* mutant and WT plants were floated in a pure-water bath at 20 °C, and the light sources were halogen lamps placed above a circulating-water tank with thermal insulation. The leaves were placed under high irradiance (HI, 1 300 μmol m^{-2} s^{-1}) and were periodically taken for particular analyses.

After centrifugation of the methanolic extracts for 2 min at 3 000 *g*, the supernatant absorbance (A_{530} - A_{657}) was quantified by UV- visible spectrophotometer *Lambda 24*, *Perkin-Elmer*, Waltham, MA, USA) according to the methods described by Wade *et al.* (2003). In addition, absorption spectra were also recorded from 400 to 700 nm. Flavonoid and total phenolics extracted from leaf pieces were analyzed and estimated following the methods of Fukumoto and Mazza (2000). Chlorophyll (Chl) content was determined in 95 % ethanol extract as described by Lichtenthaler and Wellburn (1983).

The scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined as described by Peng *et al.* (2000). In brief, 50 % ethanol extracts were prepared from the leaves, followed by centrifugation at 5 000 *g* for 15 min. The resulting supernatant was mixed with DPPH solution. The decrease in absorbance was measured at 525 nm using a spectrophotometer *Lambda 25* (*Perkin-Elmer*).

For determination of cell-membrane leakage rate, leaves were immersed in double-distilled water for 1.5 h at room temperature, followed by a 30-min boiling. The conductivity of a solution of leaked electrolytes before and after boiling was determined using a *DDS-11 A* conductometer (*Shanghai Dapu Instruments*, Shanghai, China).

ROS localization was conducted according to Romero-Puertas *et al.* (2004) and Zeng *et al.* (2010) with some modifications. After HI treatment for 0, 120 and 240 min, samples were collected for analysis of H_2O_2 and $O_2^{\bullet-}$ localization *in situ*. H_2O_2 was visualized by diamino-benzidine (DAB) staining. Samples from each treatment were immersed in a solution of DAB (1 mg cm^{-3}, *Sigma-Aldrich*, St. Louis, MI, USA) in 50 mM phosphate buffer (pH 7.0), vacuum-infiltrated for 10 min and then incubated at room temperature for 8 h in the dark. Then white light (80 μmol m^{-2} s^{-1}) was switched on 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 $O_2^{\bullet-}$ localization *in situ*, samples from each treatment were immersed in a 0.1 mg cm^{-3} solution of nitroblue tetrazolium (NBT) in 50 mM K-phosphate buffer (pH 6.4), containing 10 mM Na-azide, and then were vacuum-infiltrated for 5 - 10 min and left under white light until the appearance of dark spots, characteristic of blue formazan precipitates. The subsequent steps were the same as for localization of H_2O_2.

One set of cut leaves was placed under HI and periodically taken for Chl fluorescence measurements carried out with an *Imaging-PAM* Chl fluorometer (*Heinz Walz*, Effeltrich, Germany) as described by Siebke and Weis (1995) and Rascher *et al.* (2001). All fluorescence measurements were started after 10-min dark adaptation. When performing a measurement, an area of interest (AOI) with a diameter of 1 cm was selected in the middle of the whole leaf. Values of the Chl fluorescence parameters such as variable to maximum fluorescence ratio (F_v/F_m characterising maximum photosystem 2 efficiency), effective photosystem 2 quantum yield ($\Phi_{PS2} = 1 - F_s/F_m'$) and coefficient of photochemical quenching ($qP = (F_m' - F_s)/F_v'$) were determined. In addition, their images were simultaneously derived from the *Imaging-PAM* software. The definition and calculation of the apparent electron transport rate (ETR) were performed according to Shao *et al.* (2008). Cut leaves placed under HI at 20 °C for 0, 60 and 90 min were also used for rapid light curve measurements (Schreiber *et al.* 1997) carried out using 20-s exposures to stepwise increased irradiance (0, 1, 21, 56, 111, 186, 281, 336, 396, 461, 531, 611, 701, 801, 926, 107, 1251μmol m^{-2} s^{-1}). Simultaneously, ETR values were obtained automatically using the *ImagingWin* software.

Reported values of all measurements correspond to the

mean of three to five measurements made on three to five different plants. Student's *t*-test was used to test for statistical differences between two *Arabidopsis* phenotypes. Statistical analyses were performed with *SPSS 12.0* (*SPSS*, Chicago, IL, USA). Graphing was performed using *SigmaPlot 11.0* software (Systat Software, San Jose, CA, USA).

Results

Anthocyanins display a typical absorption peak at 530 nm. The WT showed a marked peak at 530 nm, while the *tt3tt4* anthocyanin-deficient mutant exhibited no obvious peak of absorption at 530 nm. Correspondingly, the anthocyanin content in the WT was nearly four-fold higher than in the *tt3tt4* mutant (data not shown).

After 8 h exposure to HI, there was an obvious decrease in the content of total Chl in the two *Arabidopsis* phenotypes, the *tt3tt4* mutant showing a more pronounced decrease ($P < 0.01$, Fig. 1); however, HI stress had no substantial effect on the carotenoid content. The contents of flavonoids and total phenolics in the two *Arabidopsis* phenotypes were significantly different ($P < 0.001$) prior to HI treatment (Table 1). After 3 - 9 h under HI, the content of flavonoid in the *tt3tt4* mutant decreased by 3 % only, and did not even decrease in the WT. Similarly, the content of total phenolics did not decrease in the both phenotypes. Before HI treatment, no obvious difference was found in the DPPH scavenging capacities between the leaves of *tt3tt4* mutant and WT (Table 1). After HI treatment for 9 h, the DPPH scavenging capacity decreased by 10 % in the *tt3tt4* mutant. By contrast, the DPPH scavenging capacity in the WT increased by 23 %, the increase being mostly related to the increase in the contents of precursors for the synthesis of anthocyanins. Under HI lasting 3 - 9 h, the cell-membrane permeability increased more noticeably in the leaves of the *tt3tt4* mutant as compared to the WT, thus indicating that the plasma membranes in the former suffered more damage (Table 1).

When plants are continuously exposed to HI, ROS accumulate in the cells. Here, H_2O_2 and $O_2^{\bullet-}$ were detected with DAB and NBT staining, respectively, in the leaves of both WT and *tt3tt4* mutant (Fig. 2). In H_2O_2 detection, the brown deposits are the results of the reaction of DAB with H_2O_2. In control leaves, some pale brown deposits were observed, without significant differences between WT and *tt3tt4*. As compared to the WT leaf, more brown deposits could be observed in leaves of the *tt3tt4* mutant when both were exposed to HI for 120 min and especially 240 min. In superoxide radical detection, where the blue formazan deposits were characteristic of reaction of NBT with $O_2^{\bullet-}$, the results showed a very similar pattern with the H_2O_2 detection.

In order to identify the levels of photoinhibition, false

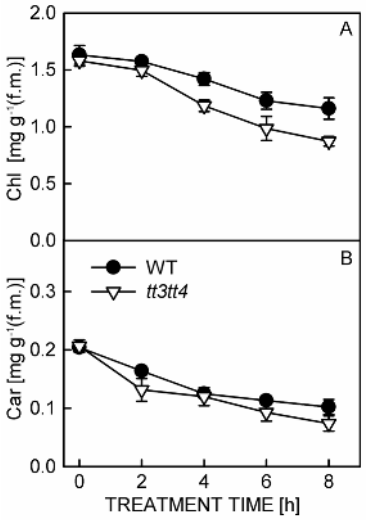

Fig. 1. Changes in the contents of chlorophylls (*A*) and carotenoids (B) in the leaves of two *Arabidopsis* phenotypes (WT: wild type; *tt3tt4*: *tt3tt4* mutant deficient in anthocyanin biosynthesis) exposed to high irradiance (1 300 μmol m^{-2} s^{-1}) for 0 - 8 h. Means ± SE, $n = 5$.

Table 1. Changes in the contents of total phenolics [ΔA_{280} mg^{-1}(f.m.)] and flavonoids [ΔA_{325} mg^{-1}(f.m.)], DPPH scavenging capacity [mg(DPPH) g^{-1}(f.m.)] and cell-membrane leakage [%] in the leaves of two *Arabidopsis* phenotypes exposed to HI (1 300 μmol m^{-2} s^{-1}) for 0, 3, 6, 9 h. Means ± SE, $n = 5$. Different letters indicate significant differences between phenotypes at the level $P \leq 0.05$.

Variables	Phenotypes	0 h	3 h	6 h	9 h
Total phenolics	WT	0.10 ± 0.00a	0.11 ± 0.00a	0.12 ± 0.00a	0.11 ± 0.00a
	tt3tt4	0.09 ± 0.00b	0.10 ± 0.00b	0.09 ± 0.00b	0.10 ± 0.00b
Flavonoid	WT	0.12 ± 0.00a	0.13 ± 0.00a	0.13 ± 0.00a	0.13 ± 0.00a
	tt3tt4	0.11 ± 0.00b	0.12 ± 0.00b	0.12 ± 0.00b	0.11 ± 0.00b
DPPH scavenging capacity	WT	0.31 ± 0.01a	0.33 ± 0.01a	0.37 ± 0.02a	0.38 ± 0.01a
	tt3tt4	0.29 ± 0.02a	0.32 ± 0.02a	0.30 ± 0.03b	0.26 ± 0.03b
Membrane leakage	WT	9.25 ± 0.25a	12.69 ± 0.21a	15.42 ± 1.35a	20.50 ± 1.34a
	tt3tt4	10.38 ± 0.53a	17.75 ± 1.23b	29.09 ± 1.60b	35.23 ± 2.82b

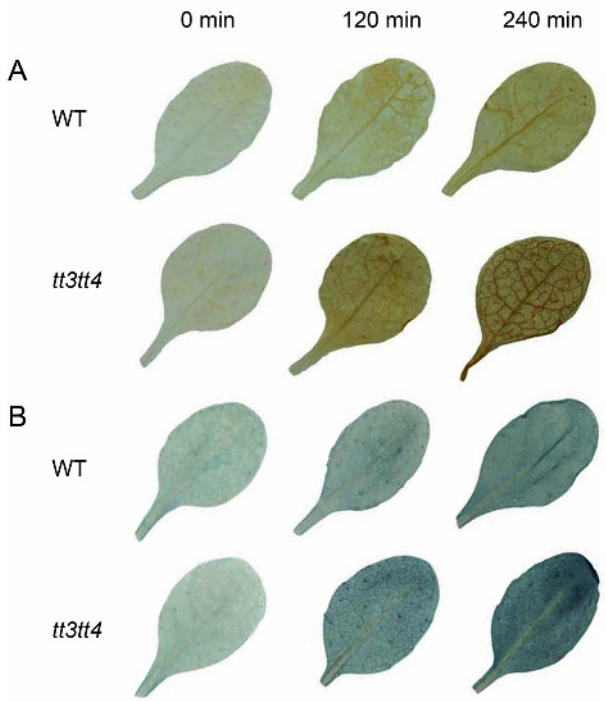

Fig. 2. H_2O_2 (*A*) and superoxide radical (*B*) localization *in situ* in the leaves of two *Arabidopsis* phenotypes (WT and *tt3tt4* mutant). The leaves were exposed to high irradiance (1 300 μmol m^{-2} s^{-1}) for time indicated, followed by infiltration with DAB or NBT for visualizing H_2O_2 and superoxide radical, respectively.

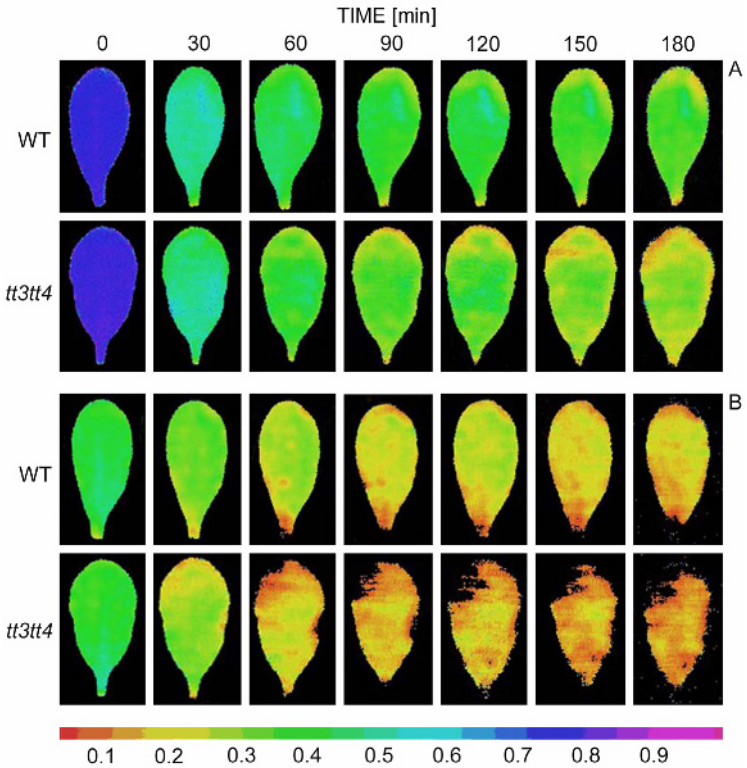

Fig. 3. Changes in the fluorescence images of the maximal PS 2 quantum yield (F_v/F_m) (*A*) and effective PS 2 quantum yield (Φ_{PS2}) (*B*) in the leaves of two *Arabidopsis* phenotypes (WT and *tt3tt4* mutant). Whole leaves were exposed to high-irradiance (1 300 μmol m^{-2} s^{-1}) for 0 - 180 min. Fluorescence images are indicated by the false colour code at the bottom. The code ranges from black *via* red, orange, yellow, green, blue and violet to purple, and these colours code for numbers between 0 and 1.

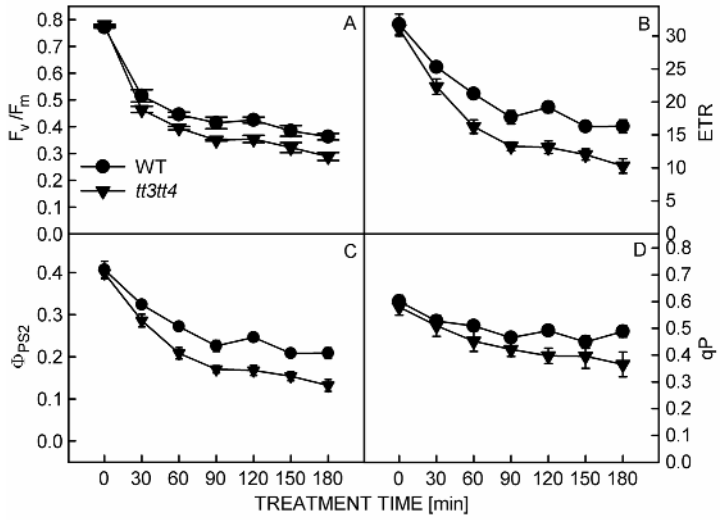

Fig. 4. Effects of high irradiance on the maximum PS 2 quantum yield (F_v/F_m) (*A*), the electron transport rate (ETR) (*B*), effective PS 2 quantum yield (Φ_{PS2}) (*C*) and the coefficient of photochemical quenching (qP) (*D*) in leaves of two *Arabidopsis* phenotypes (WT: and *tt3tt4* mutant. The whole leaves were exposed to high irradiance (1 300 μmol m^{-2} s^{-1}) for 0 -180 min. Means ± SE (n = 5).

colour images of F_v/F_m were created in our experiments (Fig. 3*A*). The distribution of F_v/F_m was not heterogeneous over the unstressed *Arabidopsis* leaf. The mean F_v/F_m value for the whole *Arabidopsis* leaf was about 0.76. After HI treatment for 180 min, the imaging colour of F_v/F_m in the leaves of WT became largely green (F_v/F_m = 0.4), although there was a small area of yellow (F_v/F_m = 0.2); by contrast, the imaging colour of F_v/F_m in the leaves of *tt3tt4* became yellow (F_v/F_m = 0.2), although there was a small area of green (F_v/F_m = 0.4). The mean Φ_{PS2} value for the whole *Arabidopsis* leaf was about 0.4 before HI treatment. After HI for 180 min, the imaging colour of Φ_{PS2} in the leaves of WT became partially yellow (Φ_{PS2} = 0.2), although there was a small area of orange (Φ_{PS2} = 0.1). However, the imaging colour in the leaves of *tt3tt4* became partially orange (Φ_{PS2} = 0.1), while there was a small area of black (Φ_{PS2} = 0; Fig. 3*B*).

Mean values of the Chl fluorescence parameters (F_v/F_m and Φ_{PS2}) from the false colour images were analysed and an obvious decreasing trend in F_v/F_m in *Arabidopsis* leaves was observed during HI treatment (Fig. 4*A*). After HI for 180 min, the values of F_v/F_m in the leaves of WT and *tt3tt4* decreased by 52.9 and 63.0 % as compared to pre-treatment levels, respectively, which indicated that *tt3tt4* mutant was more sensitive to HI stress than the WT phenotype. In addition, the same pattern also occurred in ETR (Fig. 4*B*), Φ_{PS2} (Fig. 4*C*) and qP (Fig. 4*D*).

The pattern of rapid light curve in the *tt3tt4* mutant was consistent with that of the WT phenotype, and ETR reached a maximum value when the irradiance reached ~1000 μmol m^{-2} s^{-1} (Fig. 5*A*). HI stress aggravated the decline of ETR in the two *Arabidopsis* phenotypes and markedly reduced the saturating irradiance of ETR. After treatment for 60 min, the ETR in the leaves of WT and *tt3tt4* reached a saturation value when the irradiance was ~1000 and ~900 μmol m^{-2} s^{-1}, respectively (Fig. 5*B*). After 90 min, the saturation irradiance of ETR in the leaves of *tt3tt4* decreased to ~600 μmol m^{-2} s^{-1} (Fig. 5*C*).

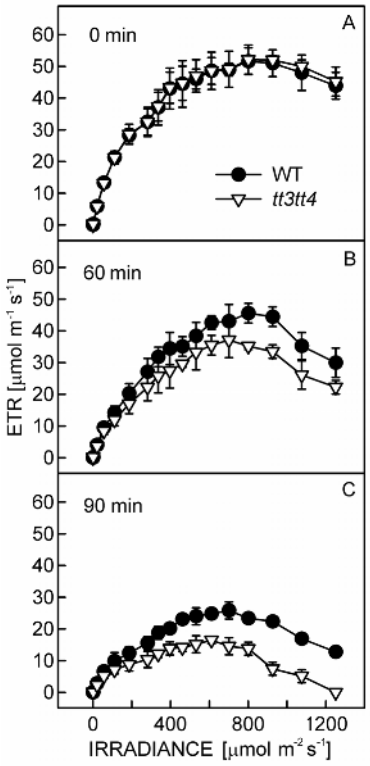

Fig. 5. Effects of different irradiances and treatment periods on the electron transport rate (ETR) in the leaves of two *Arabidopsis* phenotypes (WT and *tt3tt4* mutant). The treatment was conducted with whole leaves exposed to high irradiance of 1300 μmol m^{-2} s^{-1} for 0, 60 and 90 min, respectively. Then they were exposed (20 s) to stepwise increased irradiance (0, 1, 21, 56, 111, 186, 281, 336, 461, 531, 611, 701, 801, 926, 1076 and 1251 μmol m^{-2} s^{-1}. Means ± SE (n = 5).

Discussion

Dihydroflavonol 4-reductase (DFR) is a key enzyme in anthocyanin biosynthesis (Lu *et al*. 2010). The *tt3tt4* mutant of *Arabidopsis*, which is deficient in the DFR locus, exhibited different responses to HI exposure as compared to the WT. It is known that HI often inhibits chlorophyll biosynthesis and increases chlorophyll degradation (Hidema *et al*. 1992, Okada *et al*. 1992, Aarti *et al.* 2007, Zuluaga *et al.* 2008). The total Chl content in the *tt3tt4* mutant showed a more rapid decrease as compared to WT during HI exposure (Fig. 1*A, B*). This decrease of total Chl was mostly related to the decrease of Chl *a* (data not shown). Malondialdehyde (MDA) is an indicator of lipid peroxidation (Ding *et al*. 2010), which increases membrane leakage. The *tt3tt4* mutant and WT both exhibited increased membrane leakage; however, the former demonstrated a more obvious increase (Table 1). Long-term exposure of a green plant to HI can lead to photoinhibition (Aro *et al.* 1993) and also to production of ROS (Luo *et al.* 2010). It is obvious that the HI-treated leaves of the *tt3tt4* mutant and WT both demonstrated a reduction in PS 2 photochemical efficiency, the former suffering a greater reduction (Figs. 3, 4). In the present study, the *Arabidopsis* leaves were dark-adapted for only 30 min before Chl fluorescence measurement, and thus the mean F_v/F_m of unstressed leaves was only 0.76. It was possible that the unstressed *Arabidopsis* leaves could have a mean F_v/F_m value near 0.80 if they had been dark-adapted overnight. HI stress also led to production of ROS in the two *Arabidopsis* phenotypes, the *tt3tt4* mutant suffering a more severe oxidative stress (Fig. 2). These results indicated that the anthocyanin-deficient mutant of *Arabidopsis* was more susceptive to HI stress as compared to the WT.

We suggest that the higher resistance to HI in the WT was mainly related to the photoprotection provided by anthocyanins. As was mentioned in introduction, the anthocyanins might relieve photooxidative stress by shielding leaf tissues or by quenching ROS. The results of many studies supported the sunscreen hypothesis (*e.g*. Pietrini and Massacci 1998, Close *et al*. 2001, Albert *et al.* 2009). However, some evidence indicates that the optical properties of leaf anthocyanins may not be ideal for a sunscreen role (see review Archetti *et al.* 2009). Moreover, Gould *et al.* (2002b) reported that the effects of the anthocyanins on the penetration of blue and red radiation were negligible. Anthocyanin content in leaves of the WT was low [0.414 ± 0.061 g^{-1}(f.m.); data not shown] and the leaf colour of the WT, the same as the *tt3tt4* mutant deficient in anthocyanin, was green without appearing red. Moreover, there was no significant difference in absorption spectra between the leaves of the two *Arabidopsis* phenotypes (WT and *tt3tt4*) according to our previous research work (Shao *et al.* 2008). Hence, anthocyanins in the WT of *Arabidopsis* could not contribute substantially to shielding during HI stress in the present study. Therefore, we assumed that anthocyanins play an important role possibly as antioxidants rather than as a sunscreen.

The powerful potential antioxidative capability of anthocyanins has been extensively reported and confirmed by *in vitro* assays (Tsuda *et al.* 1996, Neil *et al.* 2002, Garcia-Alonso *et al.* 2005). A critical point to be considered in conjunction with an antioxidative hypothesis for anthocyanins concerns their localization. Anthocyanin pigments are in the vacuoles of the epidermal cells (Lee and Collins 2001), and there is no report for the presence of anthocyanins in chloroplasts. Although superoxide radicals cannot cross the tonoplast (Takahashi and Asada 1983), they are rapidly protonated to form $OH^{\bullet}$ or are converted by superoxide dismutase (SOD) to H_2O_2, which can freely penetrate the tonoplast (Takahashi and Asada 1983, Yamasaki *et al.* 1997). Less accumulation of H_2O_2 and $O_2^{\bullet -}$ was observed in leaves of the WT compared to the *tt3tt4* mutant (Fig. 2), indicating that anthocyanins can reduce ROS accumulation. In addition, the leaves of two *Arabidopsis* phenotypes showed a similar scavenging capability to DPPH before HI treatment (Table 1). The total antioxidative potential in the anthocyanin-deficient mutant might be compensated by the other antioxidants. During 0 - 9 h of HI treatment, the DPPH scavenging capacity in WT showed an increasing trend while that in *tt3tt4* showed an increasing trend followed by a decrease. We inferred that the elevated content of anthocyanins led to an increase in DPPH scavenging capacity in the WT. Our previous study reported that anthocyanins *in vitro* were more potent than ascorbic acid (AsA) against DPPH (Zeng *et al.* 2010). Moreover, stress conditions, such as HI, cold temperature, nutrient deficiency or pathogen attack, could lead to accumulations of anthocyanins in plants (*e.g*. Christie *et al.* 1994, Dixon and Paiva 1995, Albert *et al.* 2009). Hence, we suggest that it is possible that the increased precursors for the synthesis of anthocyanins in the WT can result in an increase in DPPH scavenging capacity. Anthocyanins, along with other antioxidants, may protect the leaves of *Arabidopsis* against HI-caused damage, which is consistent with HI tolerance of anthocyanin-rich leaves of a purple rice cultivar (Peng *et al.* 2006).

On the other hand, phenolics and flavonoids, like other antioxidant constituents in plant cells, are also effective ROS scavengers and play an important role in protection against membrane lipid peroxidation in plants (Chalker-Scott 1999, Zhao 1999, Shao *et al.* 2008). They reside in the central vacuole (Hutzler *et al.* 1998), yet their presence in chloroplasts has also been documented (Saunders and McClure 1976). The mutants deficient in enzymes of the anthocyanin biosynthetic pathway, chalcone synthase and dihydroflavonol 4-reductase, may have lower content of phenolics (Shao *et al.* 2008). However, the experimental results of the present study did not confirm it (Table 1).

Previous work in our laboratory confirmed that anthocyanins served as antioxidants to scavenge $O_2^{\bullet -}$ produced in PS 1 induced by methyl viologen under low

irradiance (Shao *et al*. 2008). In this study, ROS were produced in PS 2 by HI. The results provided strong indirect evidence for the role of anthocyanins in protection against free radicals and their damage to PS 2 in plants. In conclusion, anthocyanins can be used as antioxidants to extinguish ROS produced in either PS 1 or PS 2 under environmental stresses.

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