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ORIGINAL ARTICLE

The major photoprotective role of anthocyanins in leaves of *Arabidopsis thaliana* under long-term high light treatment: antioxidant or light attenuator?

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Abstract

Anthocyanins are water-soluble pigments in plants known for their photoprotective role against photoinhibitory and photooxidative damage under high light (HL). However, it remains unclear whether light-shielding or antioxidant activity plays a major role in the photoprotection exerted by anthocyanins under HL stress. To shed light on this question, we analyzed the physiological and biochemical responses to HL of three *Arabidopsis thaliana* lines (*Col*, *chi*, *ans*) with different light absorption and antioxidant characteristics. Under HL, *ans* had the highest antioxidant capacity, followed by *Col*, and finally *chi*; *Col* had the strongest light attenuation capacity, followed by *chi*, and finally *ans*. The line *ans* had weaker physiological activity of chloroplasts and more severe oxidative damage than *chi* after HL treatment. *Col* with highest photoprotection of light absorption capacity had highest resistance to HL among the three lines. The line *ans* with high antioxidant capacity could not compensate for its disadvantages in HL caused by the absence of the light-shielding function of anthocyanins. In addition, the expression level of the *Anthocyanin Synthase* (*ANS*) gene was most upregulated after HL treatment, suggesting that the conversion of colorless into colored anthocyanin precursors was necessary under HL. The contribution of anthocyanins to flavonoids, phenols, and antioxidant capacity increased in the late period of HL, suggesting that plants prefer to synthesize red anthocyanins (a group of colored antioxidants) over other colorless antioxidants to cope with HL. These experimental observations indicate that the light attenuation role of anthocyanins is more important than their antioxidant role in photoprotection.

Keywords High light stress · Anthocyanins · Photoprotection · Antioxidant · Light attenuator

Introduction

High light (HL) stress is one of the most common abiotic stresses in the growth and development of plants. The damage from HL stress does not result from the HL itself, but from the excessive absorption of light exceeding the light used for photosynthesis (Demmig-Adams and Adams 1992). When the ratio of photon flux density (PFD) to photosynthetic rate increases, it means that light is excessive and causes photoinhibition. The ratio can be increased by increasing PFD or by reducing photosynthesis efficiency under constant PFD (Demmig-Adams and Adams 1992). Therefore, HL stress often appears along with other biotic or abiotic stress. Excessive light causes accumulation of harmful reactive oxygen species (ROS). Under HL, the chloroplast is one of the main sources of ROS. The excess light energy directly induces the production of $^1O_2$ from

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Photosystem II (PSII) (Elstner 1982). Mehler reaction and photorespiration response to alleviate excess energy under HL can also cause the accumulation of by-products such as $O_2^{\cdot-}$ and H_2O_2 (Keys 1986; Clarke and Johnson 2001). ROS are known to be important signaling molecules, but excessive ROS attack intracellular components: phospholipids, proteins, nucleic acids, etc. (Alscher et al. 1997). The chloroplast, the main site of photosynthesis, is an extremely vulnerable organelle under abiotic stress (Watson et al. 2018). PSBA, PSBO-1, PSBP-1, PSBQ-1/2, and Rubisco are all oxidation targets of ROS in chloroplasts (Cruz de Carvalho 2008; Muthuramalingam et al. 2013). To cope with damage caused by HL, plants have evolved multiple strategies to protect the photosynthetic apparatus, including (i) reducing the capture of excess light, such as by leaf and chloroplast movement, epicuticular wax, non-photosynthetic pigments (Takahashi and Badger 2011); (ii) spontaneous dissipation of excess light energy captured by chloroplasts, such as by non-photochemical quenching (NPQ), which can dissipate excess absorbed light energy as thermal energy (Müller et al. 2001); and (iii) scavenging ROS by abundant enzymatic and nonenzymatic antioxidant systems (Gill and Tuteja 2010).

Anthocyanins belong to the flavonoid group of polyphenolic compounds, which are water-soluble non-photosynthetic pigments in plants (Koes et al. 2005). Chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanin synthase (ANS) are five key enzymes in the synthesis of anthocyanidins (Tanaka et al. 2008). There are great differences in the distribution, time of appearance and inducibility of anthocyanins in plant leaves. Anthocyanins can be distributed in mesophyll cells, epidermal cells, and leaf trichomes. They have a variety of functions (Zhang et al. 2016). Many studies have confirmed that, for example, under various environmental stresses, plants accumulate anthocyanins which play a certain photoprotective role in plants responding to stress (Hughes et al. 2005; Tucić et al. 2009; Liang and He 2018; Mostaka et al. 2020). So far, however, there is no uniform explanation for the significance of anthocyanins. Epidermal anthocyanins may maintain an efficient carbon-sink strength in young and senescent leaves, thus extending the leaf lifespan of red-leafed *Prunus* (Piccolo et al. 2018). The high level of anthocyanins in poinsettia leaves results in thylakoid membrane unstacking that leads to subsequent loss of PSII complexes, prevents ROS burst, maintains the activity of the remaining PSII and promotes cell survival (Mostaka et al. 2020). Aromatic hydroxyl groups and ortho-dihydroxyl groups in anthocyanins are able to inhibit free-radical chain reactions and hydroxyl radicals (Chen et al. 1996; Miguel 2011). Thus, anthocyanins play an antioxidative role by removing various types of ROS. After synthesis on the endoplasmic reticulum, anthocyanins are quickly transported through vesicles to the vacuole for storage (Poustka et al. 2007). In the acidic vacuole medium, red flavylium cations predominate and anthocyanins are red (Jurd 1963). Absorption of yellow-green light is an immutable property of all red anthocyanins, which thereby provide a sunscreen that intercepts excess light quanta that are otherwise absorbed by chlorophyll (Harborne 1958; Zhu et al.2016). Antioxidant and light attenuator are the two commonly proposed photoprotective functions of anthocyanins among all possible photoprotective functions. However, whether the main photoprotective role of anthocyanins is to confer plant antioxidative photoprotection or sunscreen photoprotection is still a hotly debated topic to date.

Some evidence supports the notion that anthocyanins participate in photoprotection as effective antioxidants. Rice-Evans et al. (1997) proposed that anthocyanins have approximately 4.4 times the antioxidant capacity of vitamin C and vitamin E. Tsuda et al. (1996) proposed that anthocyanin pigments could inhibit lipid peroxidation and scavenge ROS. Gould et al. (2002) observed that leaf cells with anthocyanins quickly remove H_2O_2. Neill and Gould (2003) demonstrated that both the colorless and red isomers of anthocyanins could eliminate $O_2^{\cdot-}$ in isolated chloroplasts of *Lactuca sativa*. Neill et al. (2002) observed that the presence of anthocyanins confers a significant antioxidant advantage to the young leaves of *Elatostema rugosum*. Kytridis and Manetas (2006) compared the protective effect of anthocyanins with different distributions in leaves under methyl viologen treatment and proposed that vacuolar anthocyanins could be an effective in vivo target for oxy-radicals. Shao et al. (2007) deemed that anthocyanins might provide photoprotection by enhancing their antioxidative capability under high-temperature stress. Xu et al. (2017) held that the ROS-scavenging role of anthocyanins helps in maintaining photosynthetic capacity to aid plant survival. These lines of evidence appear to demonstrate the antioxidative role of anthocyanins in photoprotection.

However, some researchers do not agree with this viewpoint. Anthocyanins are usually distributed in the epidermal cells of the leaves or in the vacuoles of mesophyll cells near the epidermis (Lee 2002; Poustka et al. 2007). In high light, chloroplasts, peroxisomes, and mitochondria are the main sources of ROS with no possibility of direct spatial contact with anthocyanins (Mittler 2002). More and more scholars tend to support the notion that anthocyanins mainly play a light barrier role in the process of photoprotection. Green light drives leaf photosynthesis more efficiently than red and blue light in strong white light (Terashima et al. 2009; Landi et al. 2019). Anthocyanins significantly modify the intensity and quality of light absorbed by chloroplasts by absorbing yellow-green light (Krol et al. 1995; Ntefidou and Manetas 1996). Thus, anthocyanins can reduce the risk of chloroplast damage from excess light energy and relieve chloroplast overexcitation by intercepting green light (Steyn et al. 2002).

Experiments in vitro have shown that anthocyanin solutions with concentrations higher than 0.1 mM could completely prevent the photo-transformation of photosensitive defensive chemicals in plants (Page and Towers 2002). Neill and Gould (2003) found a decline in $O_2^{\cdot-}$ generation and reduction in chlorophyll bleaching, after using a red cellulose filter, the optical properties of which approximated that of anthocyanin, to shield irradiated chloroplasts. Pietrini and Massacci (1998) used cyanidinchlorid to prepare anthocyanin solution and indirectly demonstrated that anthocyanins in corn leaves absorb about 43% of incident light. Pietrini et al. (2002) determined that HOPI lines with anthocyanin content of 8.1 μg cm^{-2} blocked 28% of incident light, but that the epidermis of W22 corn line without anthocyanin could not block incident light; thus, HOPI leaves experienced a lower risk of photoinhibition than W22. Pfündel et al. (2007) reported that anthocyanin-dependent transmittance of 50% was determined in the adaxial side or abaxial side of some autumn leaves using Dualex fluorimeter and the UV-A-PAM fluorimeter. Zhang et al. (2010) proposed that anthocyanins primarily function as light filters rather than as antioxidant molecules during HL stress in *Begonia semperflorens*. Tucić et al. (2009) held that the elevated anthocyanin concentrations in sun-exposed foliage of *Iris pumila* could act as a light attenuator, protecting its chloroplasts from excess high-energy quanta (Tucić et al. 2009). Our lab has also previously demonstrated that the major photoprotection of anthocyanins is to screen out visible radiation in *Castanopsis fissa*, *Acmena acuminatissima*, *Schima superba*, and *Cryptocarya concinna* (Zhang et al. 2016, 2018a; Zhu et al. 2018).

The aim of our present work is to further elucidate the major photoprotective role of anthocyanins under HL stress. We chose two T-DNA lines of *Arabidopsis* with different light absorption and antioxidant characteristics: *chi* and *ans*. Their responses to 200 μmol m^{-2} s^{-1} HL were compared with those of the *Columbia* wild type (*Col*). In addition, under HL, the gene expression pattern of key enzymes of the anthocyanin synthesis pathway and the pattern of contribution of anthocyanins to the antioxidant capacity were analyzed. Our data indicate that anthocyanins act more as a light attenuator than as an antioxidant in photoprotection of *Arabidopsis* plants under 200 μmol m^{-2} s^{-1} of HL.

Materials and methods

Plants and growth conditions

Arabidopsis thaliana Columbia-0 (*Col*) ecotype and all T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu/). The T-DNA insertion mutants *chi* (SALK_034145) and *ans* (SALK_073183) were in the *Col* background. CHI catalyzes the isomerization of naringenin chalcone to naringenin, which is closely related to the synthesis of flavonoids. ANS, the final key enzyme in the anthocyanin synthetic pathways, transforms colorless leucoanthocyanins into colored anthocyanidins (Tanaka et al. 2008). The T-DNA insertion mutants, *chi* and *ans*, have impaired anthocyanin synthesis. To confirm the homozygous insertion in each mutant, primer SALK LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) was used in combination with the gene-specific primers: *chi*, forward, 5′-ACGAAAACCCAACCAAATCTAAGT-3′; *chi*, reverse, 5′-AGGAACGGCGTTACCCTCTA-3′; *ans*, forward, 5′-GAAGATGGTTGCGGTTGAAAGA-3′; *ans*, reverse, 5′-ATGTGCATCACAATCGAATCAGG-3′.

Seeds were sterilized with 10% sodium hypochlorite (v/v) for 10 min, 75% ethanol (v/v) for 90 s, and washed five times with sterile double-distilled water. Seeds were synchronized in a 4 °C refrigerator for 3 days before sowing on a full strength Murashige and Skoog (MS) plate with 3% sucrose and 0.8% agar. After 10 d in MS plate, the seedlings were transferred to nutrient soil and grown in a greenhouse with 100 μmol m^{-2} s^{-1} light intensity at 22 °C in 16 h light/8 h dark cycle. To investigate the effects of HL stress on different mutants, 25-day-old plants were exposed to high light intensity of 200 μmol m^{-2} s^{-1}. Other environmental conditions except light intensity remain the same as before.

Pigment analysis

Anthocyanins were extracted in 2 mL of 1% HCl (v/v, in methanol) from 0.05 g fresh leaf at 4 °C in the dark for 24 h. Two mL of chloroform and 1 mL of distilled water were added to the extract to separate chlorophylls from anthocyanins which were dissolved in the upper water–methanol phase after blending. The absorbance of the anthocyanin extracts was recorded at 540 nm using a UV–Vis 2450 spectrophotometer (Shimadzu, Tokyo, Japan). Cyanidin-3-*O*-glucoside was used as the standard and methanol:HCl (99:1, v/v) as a blank for calculating anthocyanin concentration (Zhang et al. 2018b). At least three replicates were performed.

Chlorophylls were extracted in 2 mL of 80% acetone from fresh leaf samples (three 6 mm diameter leaf discs) at 4 °C in the dark for 24 h. The absorbance of the chlorophylls extracts was recorded at 470 nm, 645 nm and 663 nm using a UV–Vis 2450 spectrophotometer. 80% acetone was used as a blank. The chlorophyll and carotenoid concentrations were calculated according to Wellburn (1994). At least three replicates were performed.

Absorption spectra were recorded from 500 to 580 nm. Spectrophotometric analysis of whole leaves, anthocyanin extracts and chlorophylls extracts were conducted with a

UV–Vis 2450 spectrophotometer (Shao et al. 2008). Three replicates were performed.

Antioxidant capacity and phenolic content assay

A rosette leaf (0.05 g) was ground in 2 mL of 95% (v/v) methanol, and the homogenate was then centrifuged at 10,000×*g* at 4 °C for 10 min. Antioxidant capacity was measured by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) test as described by Zheng et al. (2019). Total phenolic content was measured using the Folin–Ciocalteu method according to Ainsworth and Gillespie (2007). Four replicates were performed.

Chlorophyll fluorescence measurement

Chlorophyll fluorescence measurement was carried out with a Pulse-Amplitude-Modulation (PAM) fluorometer (PAM-2100, Walz, Effeltrich, Germany). The entire *Arabidopsis* plant was dark-treated for 30 min, and then the minimal fluorescence yield of the dark-adapted state (F_o) and the maximal fluorescence yield of the dark-adapted state (F_m) of rosette leaves were measured. The maximal quantum yield of PSII photochemistry (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (Kitajima and Butler 1975). The intensity of continuous actinic illumination was adjusted to 200 μmol m^{-2} s^{-1} for 5 min. Then a saturating pulse was applied to measure the maximal fluorescence yield of the light-adapted state (F_m') and the steady-state fluorescence (F_s). Far-red light is subsequently used to record the minimal fluorescence yield of the light-adapted state (F_o'). The actual quantum yield of PSII (Y(II)) was calculated as $Y(II) = (F_m' - F_s)/F_m'$ (Genty et al. 1989). The photochemical quenching coefficient (qP) was calculated as $qP = (F_m' - F_s)/(F_m' - F_o')$ (Schreiber et al. 1986). Four replicates were performed.

Soluble protein and Rubisco content

A rosette leaf (0.05 g) was homogenized in 1 mL of protein extraction buffer (pH 7.8, containing 50 mM Tris–HCl, 20 mM $MgCl_2$, 10 mM mercaptoethanol, 10 mM PMSF and 1 mM EDTA-Na_2). After centrifugation at 13,000×*g* and 4 °C for 10 min, the soluble protein content of supernatant was determined by the Bradford method (Bradford 1976). As for determining Rubisco content, 50 μL of supernatant was mixed with 50 μL of 2 × protein loading buffer (pH 7.6, containing 10 mM Tris, 24% (v/v) glycerin, 2% (w/v) SDS, 2% (v/v) b-mercaptoethanol and 0.02% (w/v) bromophenol blue), and then incubated at 100 °C for 5 min. After SDS–PAGE electrophoresis, the Rubisco large and small chain was recognized by their molecular weight. The Rubisco protein content was estimated using TotalLab Quant software (TotalLab, Newcastle upon Tyne, UK) according standard BSA bands. Four replicates were performed.

Relative membrane leakage estimation

Three 6 mm diameter leaf discs were soaked in 5 mL of double-distilled water for 2 h at room temperature. The conductivity of the solution was measured by a DDS-11C conductometer (Shanghai Dapu Instruments) and recorded as C1. Then 5 mL of double-distilled water with leaf discs was incubated at 100 °C for 40 min, and the conductivity of leaky electrolyte was recorded as C2. Relative membrane leakage rate was calculated as C1/C2. Five replicates were performed.

$O_2^{\cdot-}$ histochemical staining

Fresh leaf samples were infiltrated with a 50 mM K-phosphate buffer (pH 6.4) containing 10 mM Na-azide and 0.1% Nitroblue tetrazolium (NBT, w/v) under vacuum for 30 min and then placed in darkness and at room temperature for 2 h. Subsequently, 95% (v/v) ethanol was used to bleach chlorophylls from the stained leaves at 100 °C. Because $O_2^{\cdot-}$ can react with NBT to produce a blue precipitate, the accumulation of $O_2^{\cdot-}$ could be observed as blue spots forming in the leaves after NBT staining (Romero-Puertas et al. 2004). Five replicates were performed.

qRT-PCR analysis of anthocyanin synthesis-related genes

Total RNA was extracted from rosette leaves using a Plant Total RNA isolation kit (Sangon Biotech). The RAN was treated with DNase I (Takara) and synthesized to complementary DNA with oligo (dT) 18 primer and the M-MLV reverse transcriptase kit (Takara). qRT-PCR was performed with SYBR Premix EX Taq II (Tli RNaseH Plus, TaKaRa). The *Tubulin* (*TUB*) gene was selected as a reference gene (for primers used, see Supplementary Table S1). qRT-PCR for gene expression involved in the anthocyanin pathway (*CHS*, *CHI*, *DFR*, *F3H*, *ANS*, *UF3GT*) was calculated with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Four replicates were performed.

Anthocyanin contribution analysis

The upper and lower epidermal surfaces were attached to a strip of masking tape. The masking tape with the lower epidermal surface was then carefully pulled away from another masking tape, peeling away the lower epidermal surface cell layer. Then, leaf discs were symmetrically punched out of the lower epidermis and leaf without lower epidermis. Using the veins as the axis of symmetry, the lower epidermis was

taken from two pairs of symmetrical leaf discs. Leaf discs were soaked in 2 mL of 95% methanol to determine the total antioxidant capacity as mentioned earlier. The symmetrical leaf discs were soaked in 2 mL of 1% HCl (v/v, in methanol). The HCl-methanol extract was measured at the wavelengths of 657, 530, 325, and 280 nm by a UV–Vis 2450 spectrophotometer.[OD530–OD657], OD325 and OD280 values were normalized for leaf disc area to represent the content of anthocyanins, flavonoids and phenols (Fukumoto and Mazza 2000; Page et al. 2012). The same was done with the leaf without lower epidermis. The anthocyanin contribution to the total antioxidant capacity was calculated using the DPPH-clearance coefficient of cyanidin-3-*O*-glucoside (4 μmol μmol^{-1}) as standard. Six replicates were performed.

Microscopy observation

To reveal the distribution of anthocyanins in the leaves of *Arabidopsis*, we sliced the leaves by hand sectioning. Cross-sections of the leaves were observed and photographed under an upright fluorescence microscope (Leica, Wetzlar, Germany).

Statistical analysis

All data were analyzed by one-way ANOVA followed by Duncan's post hoc test using IBM SPSS Statistics 19.0 (IBM, NY, USA). Sigmaplot 12.5 (Systat Software Inc., USA) was used to show the data. All data were shown as means ± standard error (SE) from measurements, and $P < 0.05$ was considered as significant differences. The index that we propose for resistance (RS) for high light stress was calculated according to Orwin and Wardle (2004). C is the control parameter measured under normal condition, and T is the experimental parameter measured under high light-treated condition. Thus, RS was calculated as $RS = 1 - 2|T - C|/(C + |T - C|)$.

Results

Phenotypes of *chi* and *ans* mutation under HL treatment

To investigate the main photoprotection function of anthocyanins under HL, we took advantage of two mutants deficient in anthocyanin synthesis. *chi* and *ans* are homozygous T-DNA insertion mutants of CHI and ANS, respectively (Supplementary Fig. S1A-C, E-G). qRT-PCR analysis determined that the *CHI* expression level of *chi* was significantly lower than that of *CHI* in *Col*, and the *ANS* expression of *ans* was significantly lower than that *ANS* in *Col* (Supplementary Fig. S1D, H). *Arabidopsis* plants were grown under normal growth conditions (100 μmol m^{-2} s^{-1}) for 28 days. Then 28-day-old plants with uniform growth were divided into two groups and cultivated under different light conditions: normal growth (100 μmol m^{-2} s^{-1}) conditions and high light (HL, 200 μmol m^{-2} s^{-1}) conditions. Under normal growth conditions, *chi* and *ans* grew well and similarly to the wild type, except for the accumulation of redness on *Col* leaves on Day 15. No obvious yellowing of leaves occurred in all three lines until Day 15 (Fig. 1a). Under HL, *ans* mutant first turned yellow on Day 9 (Supplementary Fig. S2B). Severe yellowing of leaves and even withering of leaves occurred in *ans* after 15 days HL treatment (Fig. 1b). The line *chi* showed leaf chlorosis after 12 days of HL treatment, while *Col* was chlorotic after 15 days HL treatment (Supplementary Fig. S2B). Furthermore, the leaves of *Col* turned red after 3 d HL treatment. Still, no significant redness of the leaves was observed in *chi* and *ans* till 15 days of HL treatment (Fig. 1a).

Absorption spectra in *chi* and *ans* mutation under HL treatment

The absorption spectra (500–580 nm) of methanol-HCl extracts of rosette leaves of *Col*, *chi* and *ans* were recorded. The apparent absorption peak near 540 nm confirmed that the accumulated red substance was anthocyanins (Fig. 1d). *Col* accumulated a lot of anthocyanins after 15 days of HL treatment (Fig. 1e). The anthocyanin level of *chi* was significantly lower than that of *Col* but significantly higher than that of *ans* (Fig. 1e). Also, we recorded the absorption spectra of leaves between 500 and 580 nm of *Col*, *chi* and *ans*. Similar to the absorption spectrum of anthocyanin extracts, leaves of *Col* had the highest absorbance at 500–580 nm, followed by the leaves of *chi* and then *ans* (Fig. 1c). Besides anthocyanins, chlorophyll is also the main pigment in leaves. We extracted chlorophyll with 80% acetone and performed spectral scanning. Between 500 and 580 nm, the absorbance of the chlorophyll extracts was very low, and there was no difference between three lines. The results show that chlorophyll contributed relatively little to the absorbance of leaf at 500–580 nm. The difference in absorbance of leaves between 500 and 580 nm was mainly due to the difference in anthocyanin levels (Fig. 1c–e). It is well known that the light absorption by anthocyanins allows them to act as a light attenuator in photoprotection (Zheng et al. 2019). The difference in the anthocyanin level indicates that the three lines had different photoprotection capacities. That is to say, *Col* had the highest anthocyanin content which, as a light attenuator, gave the strongest photoprotection capacity, followed by *chi*, and finally *ans*.

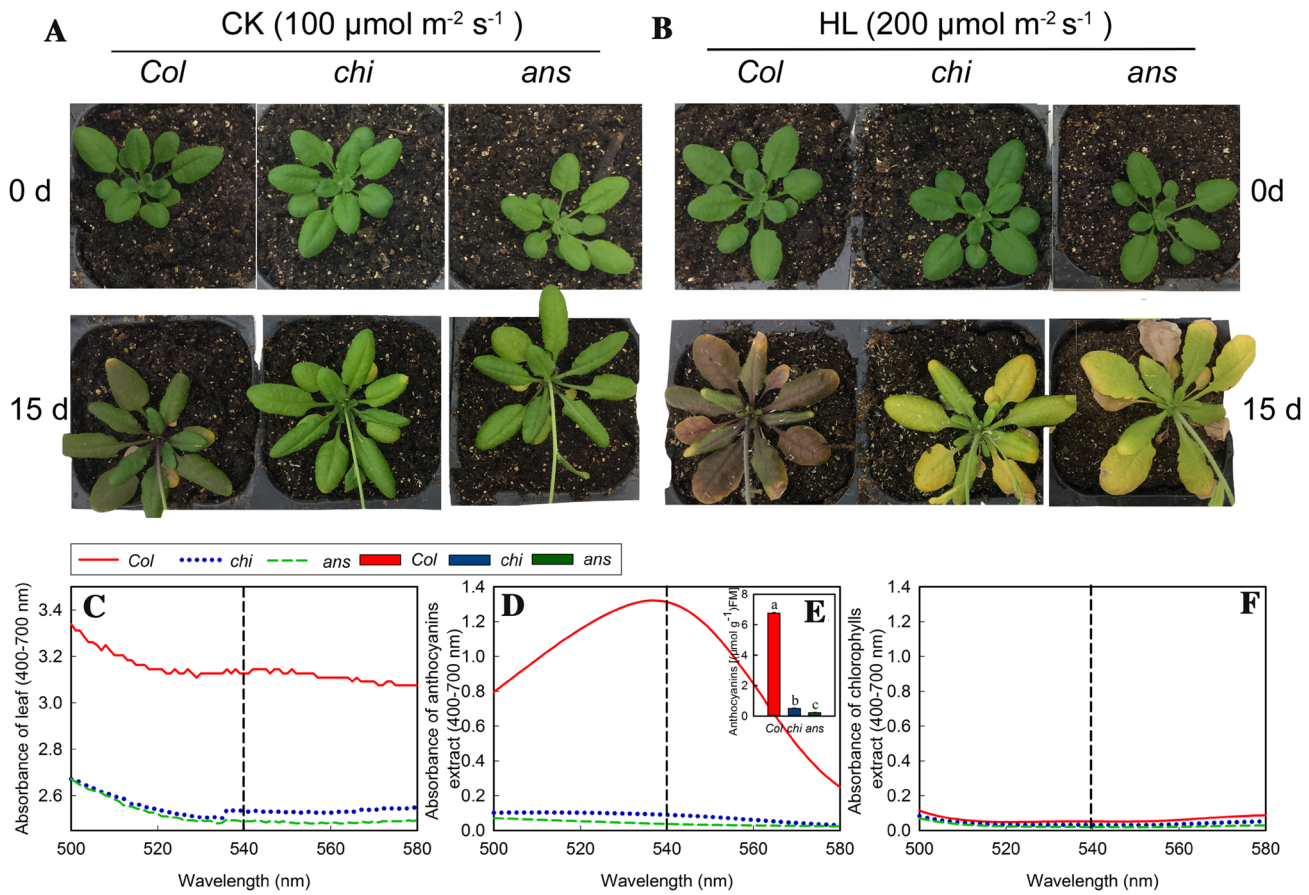

Fig. 1 Phenotypes and light absorption characteristics of the *chi* and *ans* mutation. **a**, **b** Phenotypes of 28-day-old *Col*, *chi* and *ans* plants transferred to normal light (CK, 100 μmol m^{-2} s^{-1}, **a**) and high light (HL, 200 μmol m^{-2} s^{-1}, **b**) in soil with a 16-h photoperiod for 0 day and 15 days. **c**, **d**, **f** Absorptance spectra of the leaves (**c**), anthocyanin extracts (**d**) and chlorophylls extracts (**f**) of *Col*, *chi*, and *ans* between 500 and 580 nm after 15 days HL treatment. Data are presented as means ($n=3$). **E** Anthocyanin contents of *Col*, *chi* and *ans* after 15 days HL treatment. Error bars indicate SE ($n=3$). Different letters above bars indicate statistically significant differences ($P<0.05$)

Antioxidant characterization of the *chi* and *ans* mutation under HL treatment

We measured the anthocyanin contents in the leaves of *Col*, *chi* and *ans*. Consistent with the observed phenotype, only *Col* accumulated anthocyanins under normal light (Fig. 2a). Under HL, *Col* accumulated more anthocyanins and at a faster rate than under normal light (Fig. 2a, b). The anthocyanin content of *Col* on Day 3 of HL treatment exceeded that on Day 15 under normal light (Fig. 2a, b). In addition, *chi* had significantly more anthocyanins than *ans* (Figs. 1e, 2b).

Most of intermediate products of the anthocyanin synthesis pathway belong to the flavonoid group of polyphenolic compounds (Koes et al. 2005). Thus, we also determined the phenol contents in the leaves of *Col*, *chi* and *ans*. Under normal light conditions, the phenol contents of the three lines were not significantly different and just showed a slight increase on 9th day (Fig. 2c). Under HL, the phenol contents of the three lines increased significantly from the 3rd day, especially the *ans* which accumulated the most phenols. *chi* accumulated the least phenols at the slowest rate, and the accumulation of *Col* was between *chi* and *ans* (Fig. 2d).

Then, we measured the antioxidant capacity of *Col*, *chi* and *ans* by the DPPH scavenging test. The results show that all three lines had low antioxidant capacity and no significant difference between them under normal light (Fig. 2e). Under HL, antioxidant capacity showed similar change pattern with the content of phenols (Fig. 2d, f). With the extension of HL treatment time, antioxidant capacity of three lines increased at different rates: *ans* maintained the strongest antioxidant capacity, followed by *Col*, and *chi* was the weakest one among the three lines (Fig. 2f). *ans* had almost no anthocyanin accumulation but had the strongest antioxidant capacity (Fig. 2). Thus, we treated the large amount of antioxidants accumulated in *ans* as colorless anthocyanins, that is, anthocyanins without light-filtering ability. *chi* had significantly more anthocyanins but lower antioxidant capacity than *ans*

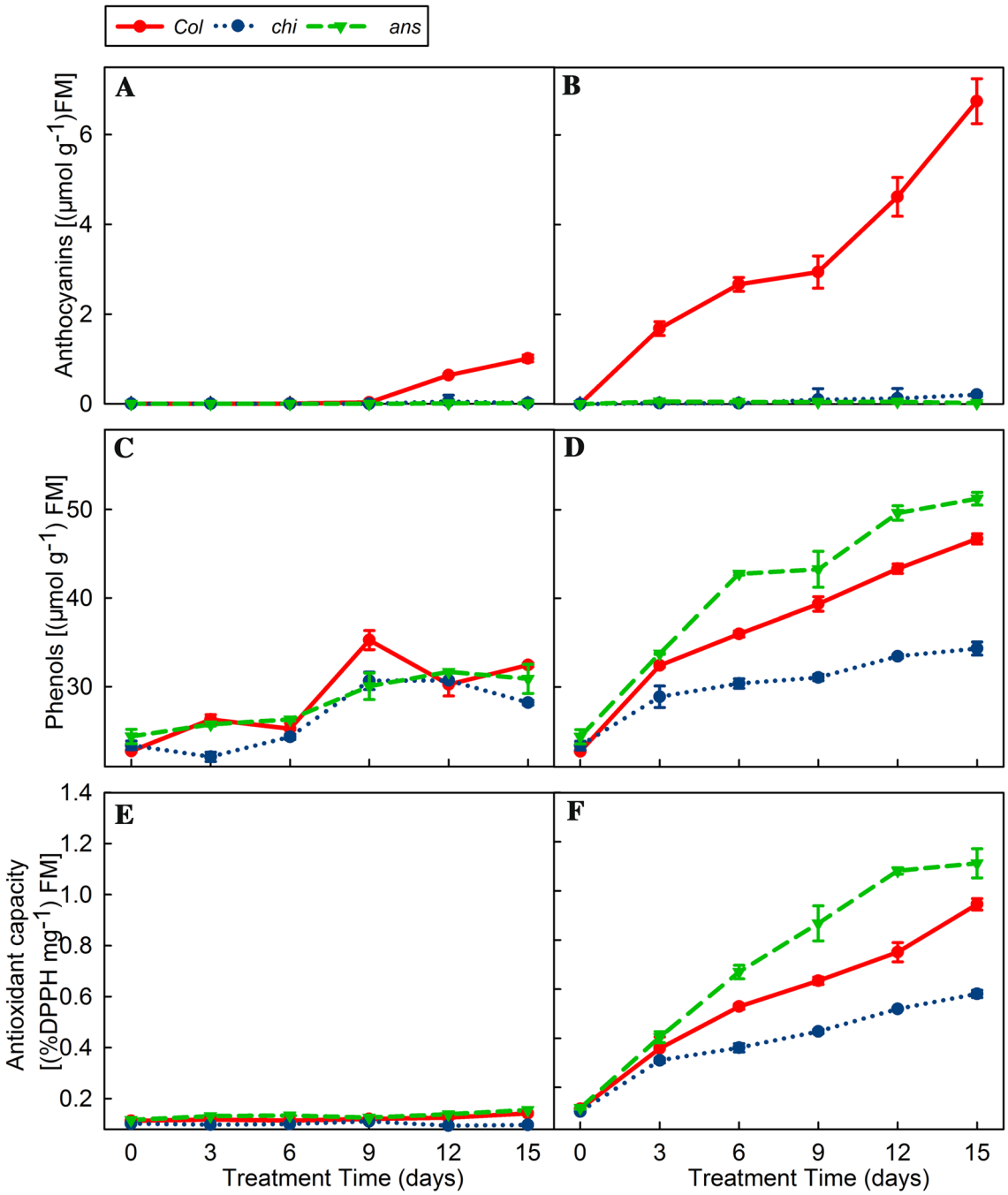

Fig. 2 Antioxidant characterization of the *chi* and *ans* mutation. **a**, **b** Changes in anthocyanin contents of *Col*, *chi* and *ans* under normal light (100 μmol m^{-2} s^{-1}, **a**) and high light (HL, 200 μmol m^{-2} s^{-1}, **b**). **c**, **d** Changes in phenols contents of *Col*, *chi* and *ans* under normal light (**c**) and HL (**d**). **e**, **f** Changes in antioxidant capacity of *Col*, *chi* and *ans* under normal light (**e**) and HL (**f**). Error bars indicate SE ($n=4$)

(Fig. 2). We treated *chi* as the plants having stronger light-attenuating photoprotection capacity but lower antioxidant capacity than *ans*. *Col* with moderate antioxidant capacity accumulated lots of anthocyanins with sufficient light attenuation and antioxidant capacity. In other words, *ans* had the highest antioxidant capacity, followed by *Col*, and finally *chi*; *Col* had the strongest light attenuation capacity, followed by *chi*, and finally *ans*. Based on their characteristics, *chi*, *ans*, as well as *Col* were suitable experimental materials to explore the dominant photoprotective functions of anthocyanin.

Changes in chloroplast physiological parameters under HL treatment

Under normal light, the chlorophyll contents of the three lines slightly increased first and began to decline until the 9th day. *Col* had the highest chlorophyll content, followed by *chi*, and *ans* was the lowest one on Day 12 and Day 15 (Fig. 3a). After 3 days of HL treatment, the chlorophyll content of *ans* firstly decreased significantly. The chlorophyll content of *chi* began to decrease on the 6th day, but remained higher than that of the *ans*. The chlorophyll content of *Col* started to decrease after 9 days HL treatment, but maintained the highest level among the three lines (Fig. 3b). Under normal light or high light treatment, the patterns of change in carotenoid content were similar with those of chlorophyll content in three lines (Fig. 3a–d).

Given the different chlorophyll contents, we measured the chlorophyll fluorescence parameters of the leaves. The results show that the F_v/F_m of all three lines remained at a high level, and did not decrease until the 12th day under normal light, even then only slightly. There was no significant difference among all three lines in F_v/F_m (Fig. 3e). The F_v/F_m of the three lines began to decrease on the 6th day of HL treatment. Among them, the F_v/F_m of *Col* decreased most slowly, and *chi* as well as *ans* decreased faster than *Col* (Fig. 3f). The trends of change in Y(II) and qP were similar to those of F_v/F_m (Fig. 3g–j). However, the Y(II) and qP of *chi* were significantly higher than those of *ans* on 15th day of HL treatment (Fig. 3h, j).

In addition to the photoreactions performed in the photosystems of the thylakoid, the carbon fixation reaction is also an important component of photosynthesis. Changes in the content of the key carbon-fixing enzyme—Rubisco—can also reflect the effect of abiotic stress on photosynthetic apparatus and photosynthesis. The results show that HL caused a lot of degradation of Rubisco. Under normal light, the Rubisco content of *Col* and *ans* was almost the same. Perhaps due to the effects of aging, the Rubisco content of *chi* was significantly lower than that of *Col* and *ans*. After HL treatment, *Col* maintained the highest Rubisco content,

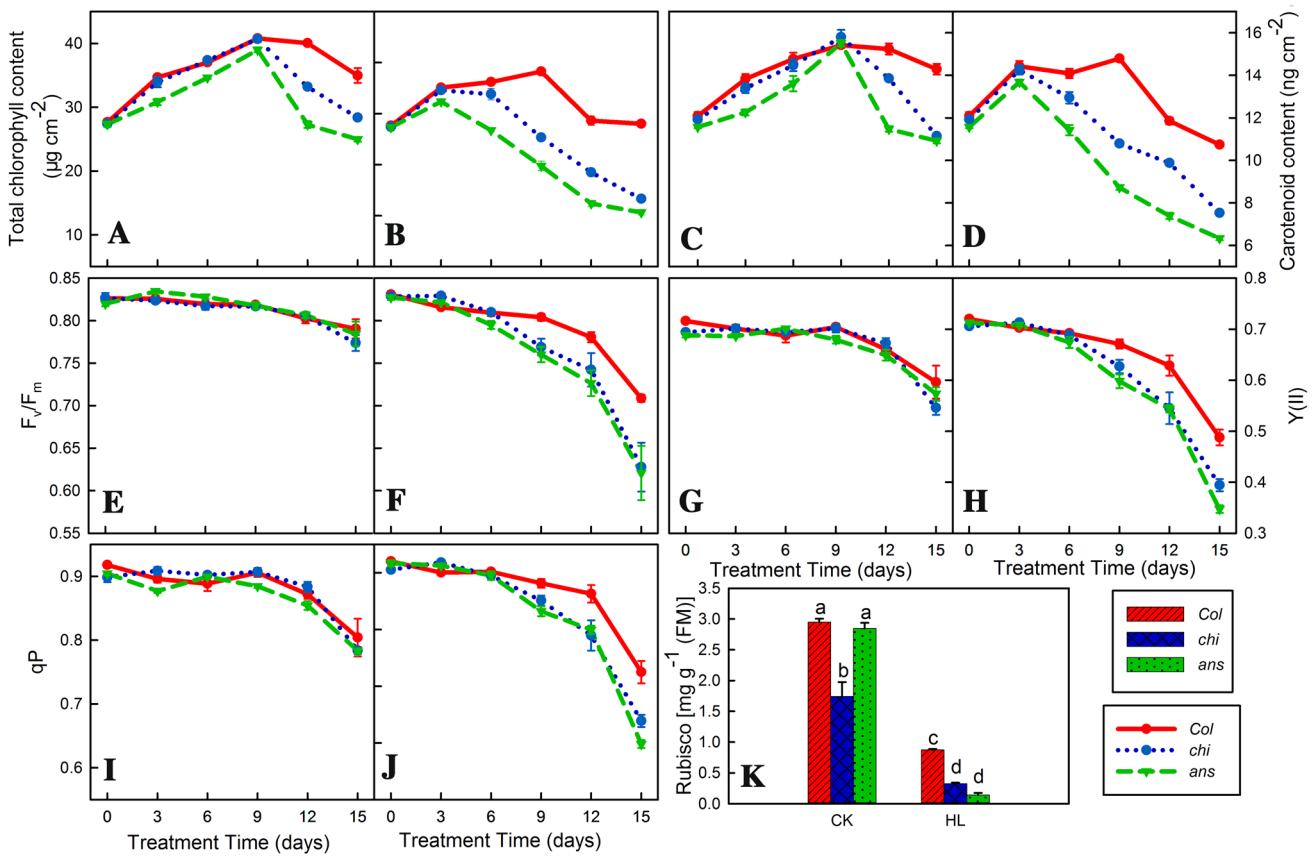

Fig. 3 Changes in different chloroplast physiological parameters of *Col*, *chi* and *ans* under normal light (CK, 100 μmol m^{-2} s^{-1}) and high light (HL, 200 μmol m^{-2} s^{-1}). **a**, **b** Changes in total chlorophyll contents of *Col*, *chi* and *ans* under CK (**a**) and HL (**b**) condition. **c**, **d** Changes in carotenoid contents of *Col*, *chi* and *ans* under CK (**c**) and HL (**d**) condition. **e**, **f** Changes in F_v/F_m of *Col*, *chi* and *ans* under CK (**e**) and HL (**f**) condition. **g**, **h** Changes in Y(II) of *Col*, *chi* and *ans* under CK (**g**) and HL (**h**) condition. **i**, **j** Changes in qP of *Col*, *chi* and *ans* under CK (**i**) and HL (**j**). **k** Rubisco contents of *Col*, *chi* and *ans* after 15 days CK and HL treatment. Error bars indicate SE ($n=4$). Different letters above bars indicate statistically significant differences ($P<0.05$)

followed by *chi*, and *ans* had the lowest Rubisco content. The Rubisco content of *Col*, *chi*, and *ans* decreased by 70.4%, 81.4%, 95.1% after HL treatment, respectively (Fig. 3k).

Oxidative damage under HL treatment

Abiotic stress often causes oxidative damage (Gill and Tuteja 2010). Using NBT histochemical staining, we detected the accumulation of $O_2^{\cdot-}$ in leaves of *Col*, *chi* and *ans*. The NBT coloring results show that under normal light, the leaves of the three lines showed a light blue color, the leaves of *chi* showing slightly more blue formazan precipitates than the other two lines (Fig. 4a). The blue formazan precipitates were distributed mainly along the veins. The accumulation of greater amounts of $O_2^{\cdot-}$ in the leaf corresponded to greater susceptibility to photodamage by HL treatment: *ans* accumulated the most blue formazan precipitates and *Col* accumulates the least in three lines. In addition, the blue formazan precipitates were not distributed along the leaf veins, but on the entire leaf surface after HL treatment (Fig. 4a).

In addition to chloroplasts, other membrane systems were also vulnerable to ROS attack. Membrane leakage was measured by detecting electrical conductivity. Under normal light, there was no significant difference among the three lines. After HL treatment, the relative membrane leakage of *Col* and *chi* increased slightly, but there was no significant difference between normal light and HL, while membrane leakage of *ans* was significantly greater than that under normal light. The membrane leakage increased by 9.4, 15.2 and 25.2% in *Col*, *chi*, and *ans*, respectively (Fig. 4b).

Resistance index under HL treatment

The different responses of the three lines under HL treatment may interfere with other environment or development factors, such as senescence. To better show the HL tolerance of three lines, we attempted to subtract the influence of other interference factors from normal light condition by calculating the resistance index of various physiological and biochemical parameters. The data showed that the resistance indices of all physiological parameters of *Col* were the highest among three strains. The resistance indices of chlorophyll *a* content, chlorophyll *a*/*b*, carotenoids content, Rubisco content, soluble protein content, and membrane leakage rate of *chi* were significantly higher than that of *ans*. Although *ans* had slightly higher resistance indices of

Table 1 Comparison of resistance index in *Col*, *chi* and *ans* under high light stress

	Col	*chi*	*ans*
Chlorophyll *a*	0.544 ± 0.011a	0.282 ± 0.017b	0.216 ± 0.011c
Chlorophyll *b*	0.706 ± 0.046a	0.431 ± 0.040b	0.434 ± 0.009b
Total Chlorophyll	0.669 ± 0.016a	0.307 ± 0.015b	0.271 ± 0.016b
Chlorophyll *a*/*b*	0.622 ± 0.020a	0.625 ± 0.006a	0.365 ± 0.011b
Carotenoids	0.602 ± 0.009a	0.510 ± 0.008b	0.409 ± 0.008c
F_v/F_m	0.813 ± 0.09a	0.688 ± 0.055ab	0.660 ± 0.056b
Y(II)	0.696 ± 0.037a	0.487 ± 0.078b	0.448 ± 0.082b
qP	0.824 ± 0.040a	0.617 ± 0.094a	0.632 ± 0.090a
Rubisco	0.174 ± 0.004a	0.102 ± 0.006b	0.025 ± 0.006c
Soluble protein	0.530 ± 0.033a	0.358 ± 0.064b	0.189 ± 0.037c
Membrane leakage	0.847 ± 0.052a	0.787 ± 0.071a	0.465 ± 0.078b

Data are means ± SE ($n = 4$). Different letters above bars indicate statistically significant differences ($P < 0.05$)

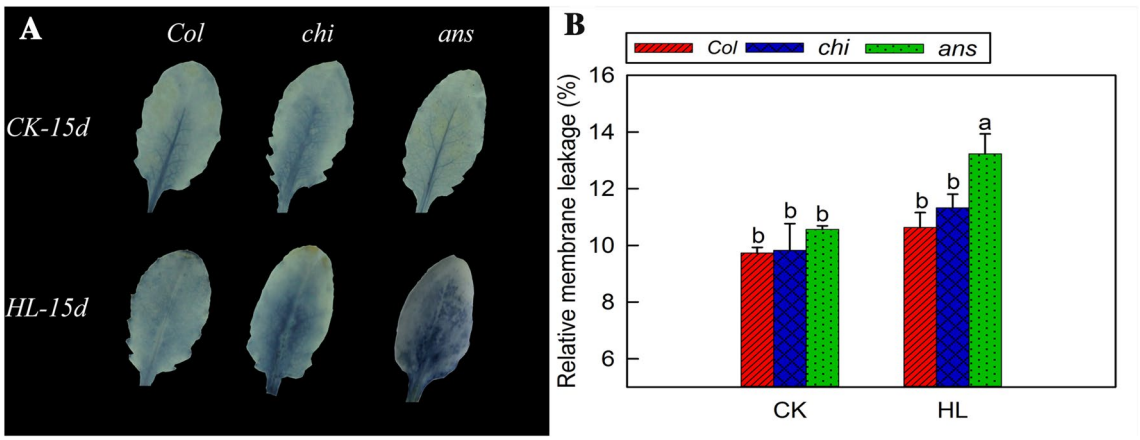

Fig. 4 Oxidative damage of *Col*, *chi* and *ans* after high light stress. **a** Accumulation of $O_2^{\cdot-}$ in the leaves of *Col*, *chi* and *ans* after 15 days normal light (CK, 100 μmol m^{-2} s^{-1}) and high light treatment (HL, 200 μmol m^{-2} s^{-1}) treatment by staining with NBT. **b** Relative membrane leakage of *Col*, *chi* and *ans* after 15 days CK and HL treatment. Error bars indicate SE ($n = 5$). Different letters above bars indicate statistically significant differences ($P < 0.05$)

chlorophyll *b* content and qP than *chi*, difference was not statistically significant (Table 1).

Changes in relative expression of anthocyanin synthetic genes under HL treatment

Given the complicated trends of anthocyanin contents and total antioxidant capacity of three lines, and also to provide new clues to the relative contributions of light attenuator and antioxidant capacity to photoprotection, we attempted to determine the gene expression levels of key enzymes of the anthocyanin synthesis pathway. After 15 days of HL treatment, the gene expression level of key enzymes of the anthocyanin synthesis pathway were significantly upregulated. The expression level of *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UF3GT* gene of *Col* after HL treatment was 6.3-, 24.8-, 2.8-, 650-, 2008-, and 103-fold as high as that of *Col* before HL, respectively (Fig. 5). After HL treatment, *ans* had significantly higher expression levels of *CHI*, *F3H*, and *DFR* genes than those of *Col*, which could explain the higher antioxidant capacity and total content of phenols of *ans* than *Col* (Fig. 5b–d). The expression of *F3H*, *DFR* and *UF3GT* of *chi* was significantly higher than that of *Col* (Fig. 5c, d, f).

Distribution of anthocyanins in *Col* leaves

By observing phenotype, we found that in the first three days after transferring *Col* to HL, the leaves began to accumulate anthocyanins. Anthocyanins did not distribute on the adaxial surface as we expected, but on the abaxial surface (Fig. 6a). The longer the HL treatment time, the more anthocyanins accumulated. From the 8th day of treatment, both the adaxial surface and abaxial surface of leaves turned red (Fig. 6b). To determine the distribution of anthocyanins induced by HL, we transected the leaves and observed the cross-section under a microscope. The microscopy results show that the anthocyanins of the leaves with red abaxial surface were mainly distributed in the lower epidermal cell layer, so we called those leaves RLE (Leaves only with red lower epidermis, Fig. 6a). Similarly, the anthocyanins of the leaves with red adaxial and abaxial surfaces were mainly distributed in the upper and lower epidermal cell layers, and we called them RFL (Red full leaf, Fig. 6b).

Analysis of anthocyanin contribution to the total antioxidant capacity in *Col* leaves

Further, we would like to find more clues to verify our ideas. We determined the correlations between antioxidant capacity and anthocyanins, and the contribution of anthocyanins to total antioxidant capacity. We used masking tape to detach the lower epidermis from the leaves, thereby obtaining the lower epidermis (LE) and the leaves without lower epidermis (mainly including the upper epidermis and most of the mesophyll cells, i.e., Mesophyll + UE). The contents of anthocyanins, flavonoids and phenols, as well as the antioxidant capacity of LE

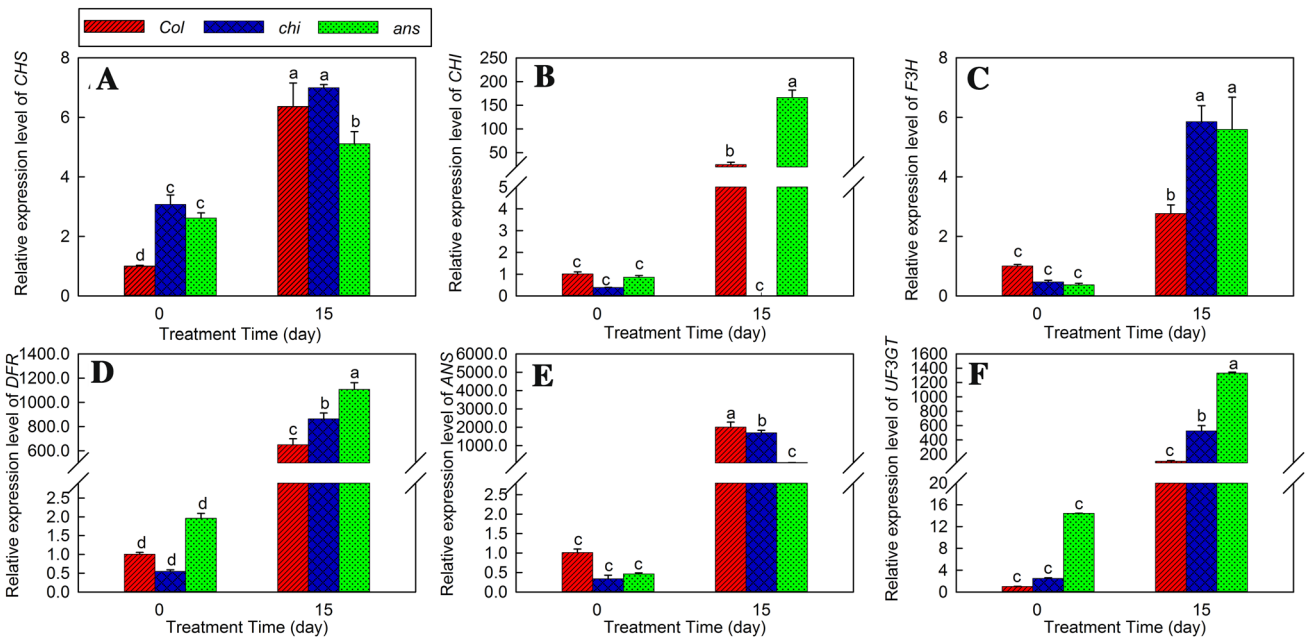

Fig. 5 Anthocyanin synthesis-related genes expression in *Col*, *chi* and *ans* under high light treatment (200 μmol m^{-2} s^{-1}). **a** *CHS*, **b** *CHI*, **c** *F3H*, **d** *DFR*, **e** *ANS* and **f** *UF3GT* gene expression level were measured at 0 day and 15 days. Error bars indicate SE ($n = 4$). Different letters above bars indicate statistically significant differences ($P < 0.05$)

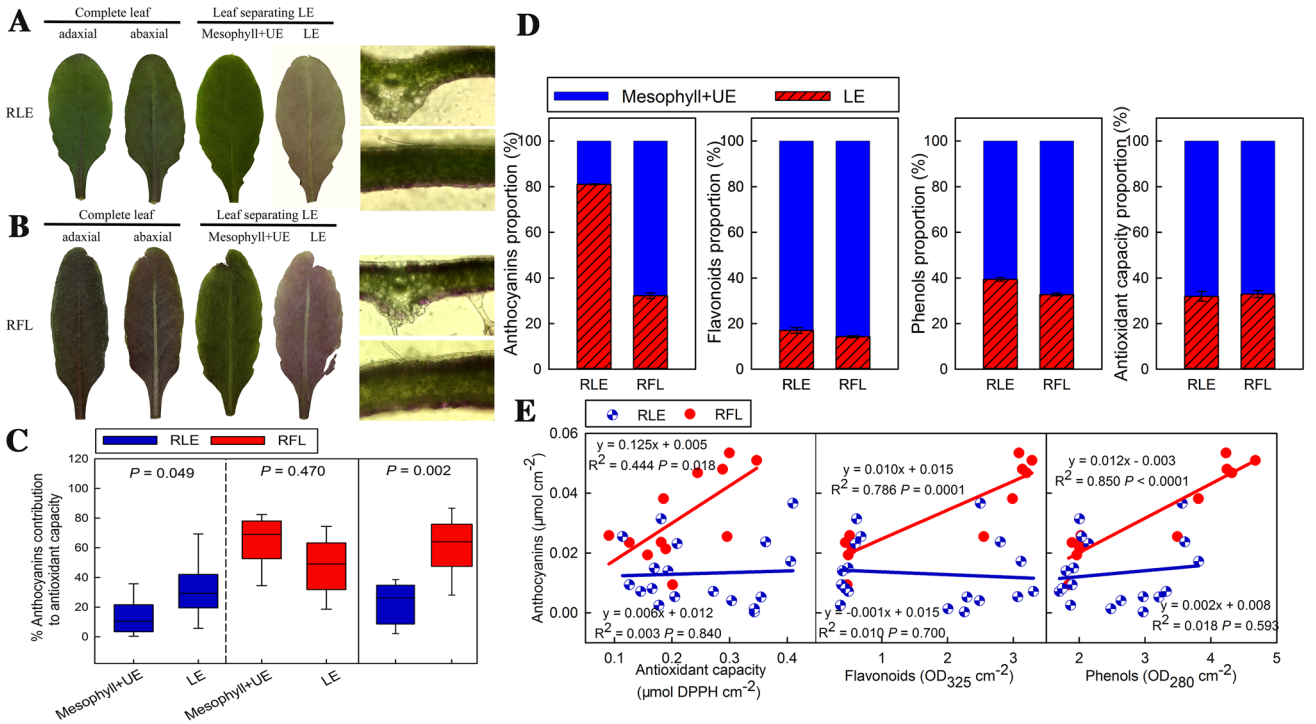

Fig. 6 Analysis of anthocyanin contribution to the total antioxidant capacity of *Col*. **a** Leaf only with red lower epidermis (RLE, before 3 days of HL treatment). The two on the left were the adaxial surface and abaxial surface of the complete leaf. The third and fourth were from the leaf after peeling away the lower epidermal (LE) surface cell layer by the masking tape. The third one was upper epidermis (UE) with most mesophyll cells. The fourth one had the LE detached. Far right were cross-sections of the complete leaf. **b** Red Full Leaf (RFL, after 8 days of HL treatment). The order was the same as RLE. **c** The anthocyanin contribution to the total antioxidant capacity in LE and UE with mesophyll cells of RLE and RFL. **d** The distribution proportion of anthocyanins, flavonoids, phenols and total antioxidant in LE and UE with mesophyll cells of RLE and RFL. Error bars indicate SE ($n = 6$). **e** Correlations between anthocyanins content and antioxidant capacity, flavonoid content and phenol content

and Mesophyll + UE were measured. To eliminate the interference of individual differences of leaves, we presented the data as the percentage of LE and Mesophyll + UE in the complete leaf. The anthocyanins of RLE and RFL had different distributions on the upper and lower epidermis, which was consistent with the results of observation of the cross-section of leaves. In RLE, the anthocyanin proportion of Mesophyll + UE was lower than that of LE, whereas in RFL the anthocyanin proportion of Mesophyll + UE was higher than that of LE (Fig. 6d, the first or leftmost panel). In both RLE and RFL leaves, the proportion of flavonoids, total phenols and antioxidant capacity of Mesophyll + UE were higher than those of LE (Fig. 6d). That is, changes in the proportion of anthocyanins in LE and Mesophyll + UE did not affect the proportion of flavonoids, phenols, and antioxidant capacity. In addition, there was no significant correlation between anthocyanin content with phenol content, flavonoid content, and antioxidant capacity in RLE leaves. By contrast, in RFL leaves treated with 8 days of HL, the content of anthocyanins showed significant positive correlation with antioxidant capacity, flavonoid content, and phenol content (Fig. 6e). Then we calculated the contribution of anthocyanins to the antioxidant capacity using the DPPH-clearance coefficient of cyanidin-3-*O*-glucoside (4 μmol μmol^{-1}) as standard (Fig. 6c). The result show that in RLE, LE had significantly higher contribution of anthocyanins to the antioxidant capacity than Mesophyll + UE (left panel, Fig. 6c), though the antioxidant capacity in Mesophyll + UE was not mainly contributed by anthocyanins in RLE. In RFL, the contribution of anthocyanin to the antioxidant capacity of Mesophyll + UE was slightly higher than that of LE, but without statistical difference (middle panel, Fig. 6c). The contribution of anthocyanins to antioxidant capacity in RLE was significantly lower than that in RFL (right panel, Fig. 6c), which means that the contribution of anthocyanins to the antioxidant capacity was gradually increased in the later stage of HL treatment. It appears that the longer the HL treatment, the greater is the contribution of anthocyanins to the pool of antioxidants.

Discussion

Anthocyanins provide significant photoprotection in HL stress, whether or not in combination with many abiotic stresses, due to their light-screening and antioxidant

properties (Neill and Gould 2003). To further understand the photoprotective mechanism of anthocyanins, much more work is needed to clarify which of these two photoprotective functions is the major function that anthocyanins play in photoprotection. Earlier, we had screened T-DNA alleles of anthocyanin biosynthesis genes in the *Arabidopsis* ecotype *Columbia*. And here, we chose two mutants with different light absorption and antioxidant characteristics: *chi* and *ans*. *chi* and *ans*, as well as *Col*, were treated under HL (200 µmol m^{-2} s^{-1}) for 15 days, and their different physiological and biochemical responses to HL were analyzed. Under HL, *ans* had the highest antioxidant capacity, followed by *Col*, and finally *chi*; *Col* had the strongest light attenuation capacity, followed by *chi*, and finally *ans* (Figs. 1, 2).

The chloroplast, which is mainly responsible for photosynthesis, is an extremely vulnerable organelle under abiotic stress (Watson et al. 2018). Chlorophylls are often degraded under HL stress (Matile et al. 1999). By absorbing quanta that would otherwise be intercepted by the chlorophylls, anthocyanins can protect chlorophylls, PSI and PSII from photoinhibitory damage and thus maintain their activities during periods of HL (Krol et al. 1995; Ntefidou and Manetas 1996). Anthocyanins, also by scavenging ROS, could possibly reduce the attack of ROS on the thylakoid membrane and photosynthetic components on membrane (Neill and Gould; Watson et al. 2018). Upon exposure to HL, the chlorophyll content of *Col*, which can normally synthesize anthocyanins, had been maintained at the highest level among the three lines (Fig. 3b). It shows that the rapid accumulation of anthocyanin alleviated chlorophyll degradation caused by HL. Chlorophyll of *ans* degraded fastest among the three lines, and the resistance index of chlorophyll *a* and chlorophyll *a/b* in *ans* was significantly lower than that of the *chi* (Fig. 3b, Table 1). With regard to chlorophyll, HL had a more negative impact on *ans* than on *chi*. In addition to chlorophyll, carotenoids are also important photosynthetic pigments. The results show that carotenoid degradation in *ans* was faster and the resistance index was lower than that of *chi* (Fig. 3d). Carotenoids are not only the basic components of photosynthetic antennas and reaction centers, but also important pigments that participate in regulating heat dissipation and provide photoprotection (Bartley and Scolnik 1995). *chi* maintained higher carotenoid content than *ans*, allowing it to provide stronger heat dissipation capacity to cope with HL stress. In addition, PSII is the critical site of damage by HL (Al-Khatib and Paulsen 1989; Allakhverdiev et al. 2008). Chlorophyll fluorescence parameters of PSII can non-intrusively reflect the effect of HL on the photosynthetic electron transfer chain on thylakoids (Krause and Weis 1991). Consistent with chlorophyll content, *Col* maintained the highest level of chlorophyll fluorescence parameters of PSII among three lines (Fig. 3); on the 15th day of HL treatment, the Y(II) and qP of *chi* were slightly higher than those of *ans* (Fig. 3h, j). Carbon fixation is also an important process of photosynthesis, and Rubisco is a key enzyme for carbon fixation (Andersson and Backlund 2008). The Rubisco content resistance index of *chi* was significantly higher than that of *ans* (Table 1). Thus, under HL treatment, the physiological activity of chloroplasts of *ans*, which lacked light-screening ability of anthocyanin but had extremely high antioxidant capacity, was weaker than that of *chi*, which had more anthocyanin accumulation but severely low antioxidant capacity.

HL stress induces a massive photogeneration of ROS from the chloroplast (Elstner 1982; Clarke and Johnson 2001). Long-lived ROS, such as H_2O_2, can be transported and detoxified by anthocyanins in the vacuole; while short-lived ROS, such as $O_2^{\cdot-}$, are rapidly protonated in the cytosol to the hydroperoxyl radical or dismutated by SOD to H_2O_2, both of which, being electrically neutral, can freely enter the vacuole and be scavenged by anthocyanins (Neill and Gould 2003). NBT-staining result shows that despite higher antioxidant capacity, *ans* accumulated more $O_2^{\bullet-}$ than *chi* (Fig. 4a). *ans* with the least accumulation of anthocyanins, had the lowest light-attenuating capacity among the three lines. Thus, under the same HL intensity, *ans* suffered higher oxidative stress than *chi* and *Col*, such that *ans* was most damaged by HL despite having an extremely high antioxidant capacity, which could have helped it to cope with the extremely high accumulation of ROS induced by HL. ROS (induced by HL) propagate out of the chloroplasts and to the cell membranes, resulting in ion leakage (Van Camp et al. 1996). After HL treatment, the membrane permeability of *Col* and *chi* increased slightly, while only the membrane permeability of *ans* increased significantly (Fig. 4b). This shows that ROS induced by HL severely damaged the cell membranes of *ans*.

All resistance indices of physiological parameters of *Col* were the highest among the three lines, suggesting the large accumulation of anthocyanins had positive significance for resisting 200 µmol m^{-2} s^{-1} of HL. *chi* had higher resistance indices of chlorophyll *a* content, chlorophyll *a/b*, carotenoid content, Rubisco content, soluble protein, and membrane leakage rate than *ans*, whereas *ans* did not have any resistance index significantly higher than that of *chi* (Table 1). This shows that although *ans* had the highest accumulation of antioxidants to alleviate the attack of ROS induced by HL, it still could not make up for the lack of light-shielding function of anthocyanins. On the other hand, although *chi* accumulated the least antioxidants, it had higher accumulation of anthocyanins and stronger light-shielding photoprotection capacity than *ans*, thereby having higher HL tolerance. Our experimental evidence, therefore, suggests that the light-shielding function of anthocyanins plays a more important role than the antioxidant function. Lacking the light-shielding function of red anthocyanins, colorless

anthocyanins with only the antioxidant capacity could not perform well in photoprotection upon HL stress.

Gene expression levels of key enzymes of the anthocyanin synthesis pathway might give us additional clues. The expression level of *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UF3GT* gene of Col after HL treatment was 6.3-, 24.8-, 2.8-, 650-, 2008-, and 103-fold as high as that of *Col* before HL, respectively (Fig. 5). Consistent with previous research, HL increases the expression of genes in the middle and late stages of anthocyanin biosynthesis (Albert et al. 2009; Xu et al. 2017; Zhang et al. 2018a). Among them, the expression of *ANS* was the most upregulated. Anthocyanin synthetase (ANS) catalyzes the conversion of colorless leucoanthocyanins into colored anthocyanidins, the precursors of anthocyanins (Springob et al. 2003). This result indicates that the transition from colorless to colored anthocyanin precursors is of great significance in HL stress. Zhang et al. (2018a) considered that under HL, *UF3GT* might be a key target of the plastid signal generated by redox imbalance to activate anthocyanin biosynthesis, and that the upregulation of *UF3GT* is a necessary and sufficient condition for leaf redness. *UF3GT* is another key enzyme downstream of *ANS* in the anthocyanin biosynthesis pathway, which catalyzes the combination of anthocyanidin and UDP-glucose into stable and water-soluble anthocyanins (Springob et al. 2003). Xu et al. (2017) proposed that ROS are an important source signal to induce anthocyanin accumulation by upregulating late biosynthetic genes. Therefore, ROS might be the important signal that induced the biosynthesis of stable and colored anthocyanins, the stability and color of which are necessary in ameliorating HL stress. Evidence based on the regulation pattern of genes expression further suggests that the light-shielding function of anthocyanins play a very important role in photoprotection.

To further reveal the major role of anthocyanins in photoprotection, we analyzed the contents of anthocyanins, flavonoids and phenols, as well as the antioxidant capacity of *Col*. In the RLE leaf up to three days of HL treatment, there was no correlation between anthocyanin content on the one hand and phenol content, flavonoids content or antioxidant capacity on the other. However, in RFL, in which anthocyanins appeared on both upper and lower epidermis in the later stage of HL treatment, the anthocyanin content was significantly and positively related to flavonoid content, phenol content and antioxidant capacity (Fig. 6e). This observation shows the important addition of anthocyanins to phenols and flavonoids to form a large antioxidant pool in the later stage of HL treatment (after 8 days of HL treatment). The increased contribution of anthocyanins to the antioxidant capacity in the later stage of HL treatment further confirms this idea (Fig. 6c). Interestingly, the difference in the proportion of anthocyanins in LE and Mesophyll + UE of RLE and RFL did not affect the proportion of flavonoids, phenols, and antioxidant capacity (Fig. 6d). Thus, our experimental evidence shows that under HL, *Arabidopsis* plants preferred to synthesize red anthocyanins, a group of colored flavonoids, rather than other colorless flavonoids with only antioxidant capacity in the upstream of biosynthesis of anthocyanins. The light-shielding function of anthocyanins played a more important role in photoprotection than did their antioxidation function. The light-shielding function of anthocyanins was to reduce the excess light energy, a smart strategy by reducing the damage at light source. The antioxidative function of anthocyanins offers photoprotection after the formation of ROS brought by HL and before oxidative damage. Plants make a trade-off between growth and resistance to environmental stress. If the antioxidant effect of anthocyanins plays a major role in photoprotection, why do plants not use other colorless flavonoids in the upstream of anthocyanin biosynthesis, but spend substances and energy to synthesize anthocyanins which function as a light attenuator? Consuming more substances and energy to synthesize anthocyanins implies that in addition to the antioxidant function of anthocyanins, the light-filtering function of anthocyanins is more urgent and more important in coping with HL.

Micrographs of cross-sections of *Arabidopsis* leaves show that anthocyanins tended to be distributed in the epidermis and the adjacent mesophyll cells during HL treatment (Fig. 6a, b). Anthocyanins can be distributed in mesophyll cells, epidermal cells, leaf trichomes, and have a variety of functions (Zhang et al. 2016). The near epidermal distribution of anthocyanins may provide greater photoprotection by the pigments acting as a light attenuator. It is well known that absorption of yellow-green light is an invariant property of all red anthocyanins (Harborne 1958). Green light is an important driving force for photosynthesis, especially in the lower mesophyll tissue (Neill and Gould 2003; Terashima et al. 2009; Landi et al. 2019). Red anthocyanins can filter out excess light of yellow-green wavelengths. The presence of red anthocyanins, which are usually stored in the vacuoles of cells in red leaves, will result in fewer photons reaching the chloroplast than in green leaves (Gould et al. 2018). However, in the initial phase (up to three days of HL treatment), anthocyanins were mainly distributed in the lower epidermal cell layer of the leaf (Fig. 6a). How do abaxial anthocyanins play a photoprotective role in attenuating light? It has been reported that light, particularly green light, does not travel in the leaves in a linear path, but bounces back and forth within the leaf lamina (Vogelmann and Evans 2002; Landi et al. 2019). Therefore, the ability to attenuate green light is largely independent of the distribution of anthocyanins (Neill and Gould 2000). Further, there is an advantage associated with abaxial location of anthocyanins: Hughes et al. (2014) proposed that adaxial anthocyanins predispose tissues to increased shade acclimation and, consequently, reduced photosynthetic capacity,

while abaxial anthocyanins may be a compromise between costs/benefits. With the extension of HL treatment time, the continuous high-intensity light promoted more synthesis of anthocyanins in adaxial tissues to filter excess light energy (Fig. 6b). Adaxial/abaxial anthocyanin plasticity may, therefore, be adaptive in HL environments (Hughes and Smith 2007). Anthocyanin distribution in adaxial leaf tissues is a more economical strategy: light incident on the leaf surface is directly intercepted, and excess light energy is filtered out by adaxial anthocyanins. Thus, anthocyanins in adaxial leaf tissues provide greater photoprotection than in abaxial tissues (Hughes et al. 2014).

Under HL treatment, the HL tolerance of *ans*, which lacked light-filtering ability of anthocyanins but had extremely high antioxidant capacity, was weaker than that of *chi*, which had more anthocyanin accumulation but severely low antioxidant capacity. HL upregulated the expression of genes in the middle and late stages of anthocyanin biosynthesis, especially *ANS* which catalyzed the conversion of colorless leucoanthocyanins into colored anthocyanidins. The conversion of colorless into colored anthocyanin precursors was necessary under HL stress. The near epidermal distribution of anthocyanins provided greater photoprotection via light attenuation. The contribution of anthocyanins to antioxidant capacity increased in RFL in the late period of HL. Under HL, plants preferred to synthesize red anthocyanins, a group of colored flavonoids, rather than other colorless flavonoids. In short, light attenuation plays a more important role than antioxidant activity in the photoprotection facilitated by anthocyanins under 200 μmol m^{-2} $s^{-1}$of HL. Interestingly, *ans* had similar tolerance to *Col* and higher tolerance than *chi* under 1600 μmol m^{-2} s^{-1} of HL for 4 h (unpublished data). We propose that the main photoprotection of anthocyanins is related to the intensity and duration of HL. If this idea is true, what directly decodes a stimulus of different intensity and duration of HL into a cellular signal, and what induces cells to accumulate anthocyanins? Further investigation is, therefore, required to decide in favor of or against the conjecture and to better explain the main photoprotective role of anthocyanins.

Author contributions All authors contributed to the study conception and design. C-LP conceived the idea and designed the experiments. X-TZ performed most of the experiments. Z-CY, Y-LC, J-WT, and M-LC provided technical assistance. X-TZ and Z-CY analyzed the data; X-TZ, Z-CY, and C-LP wrote the manuscript. C-W Y and W-S C corrected the manuscript. All the authors approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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