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ORIGINAL ARTICLE

Reactive oxygen species produced via plasma membrane NADPH oxidase regulate anthocyanin synthesis in apple peel

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Abstract
Main conclusion **Solar ultraviolet irradiation regulates anthocyanin synthesis in apple peel by modulating the production of reactive oxygen species via plasma membrane NADPH oxidase instead of other pathways.** The synthesis of anthocyanin in apple peels is dependent upon solar irradiation. Using 3-mm commercial glass to attenuate solar UV-A and UV-B light, we confirmed that solar UV irradiation regulated anthocyanin synthesis in apple peels after exposing previously bagged fruit to sunlight. During sunlight exposure, UV attenuation did not affect the expression of *MdHY5, MdCOP1,* or *MdCRY2*, but significantly lowered plasma membrane NADPH oxidase activity and superoxide anion concentrations. UV attenuation also reduced the expression levels of *MdMYB10*, *MdPAL, MdCHS, MdF3H, MdDFR*, *MdANS* and *MdUFGT1*, UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT) activity, and local concentrations of anthocyanin and quercetin-3-glycoside. In contrast, exogenous application of hydrogen peroxide could enhance anthocyanin and quercetin-3-glycoside synthesis. Xanthophyll cycle pool size on a chlorophyll basis was higher but its de-epoxidation was lower under direct sunlight irradiation than that under UV-attenuating conditions. This suggests that reactive oxygen species (ROS) produced in chloroplast are not major contributors to anthocyanin synthesis regulation. Inhibition of plasma membrane NADPH oxidase activity lowered the production of ROS through this mechanism, significantly inhibited the synthesis of anthocyanin, and increased the total production of ROS in apple peel under direct sunlight irradiation, suggesting that ROS produced via plasma membrane NADPH oxidase regulates anthocyanin synthesis. In summary, solar UV irradiation regulated anthocyanin synthesis in apple peels by modulating the production of ROS via plasma membrane NADPH oxidase.

Keywords Anthocyanin · Apple peel · Plasma membrane NADPH oxidase · Reactive oxygen species · Solar irradiation · UV-B

Abbreviations

A	Antheraxanthin
ANS	Anthocyanidin synthase
APX	Ascorbate peroxidase
CHS	Chalcone synthase
COP1	The E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1
CRY2	Cryptochrome2
DFR	Dihydroflavonol 4-reductase
DHAR	Dehydroascorbate reductase
DPI	Diphenyleneiodonium chloride
F3H	Flavanone-3-hydroxylase
GR	Glutathione reductase
HY5	The bZIP transcription factor ELONGATED HYPOGOTYL 5

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MAPK	Mitogen-activated protein kinase
MDHAR	Monodehydroascorbate reductase
PAL	Phenylalanine ammonia lyase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UFGT	UDP-glycose:flavonoid 3-O-glycosyltransferase
UVR8	UV RESISTANCE LOCUS 8
V	Violaxanthin
Z	Zeaxanthin

Introduction

Anthocyanins belong to a group of water-soluble pigments responsible for the red, purple, and blue colors of many fruits, vegetables, flowers and other plant tissues. This essential pigment plays a photoprotective role in plants under high levels of light or other stress conditions (Williams et al. 2003; Hughes et al. 2005, 2012). The photoprotective function of anthocyanin is generally attributed to two mechanisms: shielding the chloroplast from excess light by absorbing blue–green light (Smillie and Hetherington 1999; Merzlyak et al. 2008) and directly scavenging reactive oxygen species (ROS; Gould et al. 2002; Neill and Gould 2003; Bi et al. 2014). Anthocyanin has also been shown to extend the shelf life of tomato fruit (Bassolino et al. 2013), and act as an antioxidant to reduce oxidative DNA damage, as observed in rats and humans (Ramirez-Tortosa et al. 2001; Weisel et al. 2006).

The synthesis of anthocyanin has been studied extensively in plants. It is suggested that anthocyanin synthesis in fruit peel is mainly controlled by MYB transcription factors including MYB1, MYBA, and MYB10 (Allan et al. 2008; Lin-Wang et al. 2010). Among their allelic homologs, MYB1 and MYBA share identical sequences (Ban et al. 2007), while MYB10 and MYB1 genes are located at very similar positions on linkage group 9 of the apple genetic map (Chagné et al. 2008). In apple fruit, it was reported that *MdMYB10* might control both skin color and anthocyanin accumulation in fruit flesh in certain apple genotypes, whereas *MdMYB1* and *MdMYBA* were expressed in red sections of fruit skin in other genotypes (Takos et al. 2006; Ban et al. 2007; Espley et al. 2007, 2009). Compared to that of non-red fruits, red apple and pear fruit peels showed higher levels of MYB transcription (Takos et al. 2006; Ban et al. 2007; Feng et al. 2010; Lin-Wang et al. 2011; Li et al. 2014). In these fruits, the regulation of anthocyanin synthesis by MYB was mainly correlated with UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT), an enzyme which catalyzes the final step of anthocyanin synthesis. Other enzymes such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone-flavanone isomerase (CHI), flavanone-3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) are also involved in the synthesis of anthocyanin in apple or pear peels (Li et al. 2013a, 2014). *MYB* (*MdMYB1*, Py*MYB10,* and *MdMYBA*) and *UFGT* levels, UFGT activity, and anthocyanin concentration can be enhanced by sunlight (Takos et al. 2006; Li et al. 2008, 2012, 2013a, 2014; Yu et al. 2012) or UV irradiation (Ubi et al. 2006; Ban et al. 2007; Xie et al. 2012) in apple or pear peels, indicating that anthocyanin synthesis is regulated by light. However, the mechanisms underlying sunlight-mediated MYB and anthocyanin synthesis are unknown.

As the regulatory effects of both UV-A and UV-B on anthocyanin synthesis have been observed in leaves (Tossi et al. 2011; Wang et al. 2012), fruit peels (Ubi et al. 2006; Hagen et al. 2007; Xie et al. 2012), and cell cultures (Hirner et al. 2001), it is possible that sunlight regulates MYB expression through UV-A and/or UV-B rays. To date, two classes of blue/UV-A light photoreceptors (phototropins and cryptochromes, Lin 2000; Briggs and Christie 2002), and one UV-B light receptor (UV RESISTANCE LOCUS 8, UVR8, Brown et al. 2005; Rizzini et al. 2011; Christie et al. 2012) have been identified. Meanwhile, two key regulators of UV-B signal transduction have also been identified: the bZIP transcription factor ELONGATED HYPOGOTYL 5 (HY5) and the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1, Brown and Jenkins 2008; Favory et al. 2009; Jenkins 2009; Jaakola 2013). In Arabidopsis, Rizzini et al. (2011) demonstrated that UVR8 monomerizes in a UV-B-dependent manner before interaction with COP1. Li et al. (2012) found that MYB1 protein in apple peel accumulated in light conditions but degraded via a ubiquitin-dependent pathway in the dark, which was regulated by COP1. Another study of apple peel suggested that UV-B could induce the accumulation of anthocyanin via COP1-mediated signaling, leading to activation and binding of HY5 to the promoter regions of MYB genes (Peng et al. 2013). In addition, a blue/UV-A light receptor gene *MdCRY2* from apple was shown to regulate anthocyanin synthesis in Arabidopsis flower (Li et al. 2013b). These studies indicate that UV-A and/or UV-B light receptors might be involved in the regulation of MYB transcription factor and anthocyanin synthesis in plants. However, a UVR8-independent UV-B signaling pathway has also been reported in plants (Brown and Jenkins 2008; González Besteiro et al. 2011). It is suggested that narrow-band UV-B treatment could lead to the up-regulation of the UVR8 signaling pathway, whereas broad-band higher fluence UV-B treatment may be more likely to activate UVR8-independent signaling

pathways such as ROS, DNA damage, phytohormones, and mitogen-activated protein kinases (MAPKs, Wargent and Jordan 2013).

Recent studies found that ROS might be involved in the regulation of anthocyanin synthesis in apple peels under sunlight (Chen et al. 2013a; Bi et al. 2014). In plants, the production of ROS is governed by photosynthetic electron transport, photorespiration, respiration, plasma membrane NADPH oxidase, and other production systems (Asada 1999; Foyer and Noctor 2000; Apel and Hirt 2004; Sagi and Fluhr 2006). Among these systems, plasma membrane NADPH oxidase plays a key role. ROS produced by plasma membrane NADPH oxidase can serve as signal molecules to regulate plant senescence (Chen et al. 2013b), nodule functioning (Marino et al. 2011), root hair elongation (Foreman et al. 2003), and biotic or abiotic stress defense responses (Orozco-Cárdenas et al. 2001; Sagi and Fluhr 2001; Torres et al. 2002; Xia et al. 2009). Moreover, the activity of plasma membrane NADPH oxidase can be enhanced by UV-B irradiation (Tossi et al. 2009). Because the photosynthetic capacity of fruit peels is very low (Chen and Cheng 2007; Li and Cheng 2008; Li et al. 2014), ROS may be largely produced in peel chloroplasts. Mubarakshina et al. (2010) suggest that ROS produced in chloroplasts, such as hydrogen peroxide, can also serve as signaling molecules. To date, however, the mechanism by which ROS production may regulate anthocyanin synthesis in apple peels is unknown. A better understanding of this process may help growers improve or control fruit color in the future. In this study, the ROS-mediated regulation of anthocyanin synthesis in apple peels was investigated under sunlight irradiation to further illustrate the light-dependent mechanisms of anthocyanin synthesis in fruit peels.

Materials and methods

Plant materials

Nineteen-year-old apple (*Malus domestica* Borkh. 'Golden Delicious') trees on M111 rootstocks were used in this study. The trees were grown at a spacing of 3 × 4 m in north–south rows in Luochuan (35.765N, 109.442E; elevation 1,033 m), Shaanxi, China. The trees were approximately 4 m in height with a central leader, and were grown under standard horticultural practices with disease and pest control. Crop load was controlled at 8 fruits per trunk cross-sectional area (cm^2). On May 10 2012, fruits were bagged with light impermeable double layer paper bags. To reinforce protection from light, the outer layer of each bag is colored yellow on the outside and black on the inside. The inner layer of each bag is red. During sunny afternoon periods, the peel temperature of bagged fruits was 3–6 °C lower than that of unbagged fruits. On August 31 2012, approximately 130 days after full bloom, five replicates of the bagged fruits were sampled (three trees per replicate, fifteen trees total) without removing the bags, to avoid exposure to light before chemical and short-term sunlight exposure treatments.

Chemical and short-term sunlight exposure treatments

Three experiments were carried out on the fruit: time course, hydrogen peroxide treatment, and diphenyleneiodonium chloride (DPI, from Sigma-Aldrich, St. Louis, MO, USA) treatment. For hydrogen peroxide and DPI treatments, fruits were immersed in 0, 2.5, 10, 50, and 150 mM hydrogen peroxide or 0, 10, and 50 μM DPI solutions overnight before sunlight exposure.

Before sunrise, fruits were removed from their bags or respective solutions and placed onto four layers of wet gauze in an open space. For UV alleviation treatment, 3-mm commercial glass ($Na_2O{\cdot}CaO{\cdot}6SiO_2$) was used to filter solar UV-A and UV-B irradiation, with 20 cm ground clearance. The glass did not noticeably change the visible range of sunlight, but transmitted light had an absorption spectrum from 300–400 nm at the UV range (Online Resource 1). For time course treatment, peels from the sun-exposed part of apples were collected approximately 1, 3, 5, and 7 days after sunlight exposure. For hydrogen peroxide treatment, sun-exposed peels were collected after 3 days exposure. After DPI treatment, sun-exposed peels were collected after 5 days exposure. During the experiment, maximal sunlight intensity was 1,200 μmol m^{-2} s^{-1} on the first day due to cloudy conditions, and 1,800 μmol m^{-2} s^{-1} on the remaining days. Peel temperature and air humidity were similar between the two different light treatments, with a maximal peel temperature of 35 ± 1 °C and minimal relative air humidity of 45 % at midday. For each of the three experiments, samples at day 0 (i.e. before sunlight exposure) were also taken. Fruit peel samples (~1 mm thick) were collected with a peeler. For each treatment at each sampling date, 20 fruits per replicate were sampled. The samples were immediately frozen in liquid nitrogen, ground into powder and mixed in liquid nitrogen with an A11 grinder from IKA® Works (VWR, Radnor, PA, USA), and stored at −80 °C until analysis.

Pigment and phenolic compound analyses

Chlorophyll compounds in fruit peels were extracted with 80 % acetone and analyzed using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan), according to Arnon

(1949). The extraction and analysis of xanthophyll cycle pigments were carried out as described by Li and Cheng (2008). Concentrations of the xanthophyll cycle pigments violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) were determined based on peak area and a calibration curve derived from an authentic standard of Z from Sigma-Aldrich.

Phenolic compounds were assayed as described by Chen et al. (2013a). The concentration of individual phenolic compounds was determined based on peak area and calibration curves derived from corresponding authentic phenolic compounds.

Enzyme activity, antioxidant metabolite, and ROS analyses

Plasma membranes were prepared, according to Sagi and Fluhr (2001), with some modifications. Briefly, fruit peel samples were ground using a mortar and pestle in buffer (5 ml g^{-1} fresh weight) containing 100 mM Tris-Mes (pH 7.8), 0.33 M sucrose, 3 mM ethylenediaminetetraacetic acid, 5 mM dithiothreitol (DTT), 10 mM ascorbate, 0.1 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 % glycerol, 0.4 % casein, 0.5 % bovine serum albumin, and 0.2 % (w/v) polyvinylpyrrolidone. The homogenate was filtered through four layers of cheese cloth, and the filtrate was centrifuged at 10,000*g* for 20 min. Microsomes were pelleted from the supernatant by centrifugation at 140,000*g* for 45 min and re-suspended in 5 mM potassium phosphate buffer (pH 7.8) containing 0.33 M sucrose and 3 mM KCl. The suspension was then fractionated by the aqueous two-phase partitioning method (Larsson et al. 1987). Phase separations were carried out in a series of 10-g phase systems with a final composition of 6.2 % (w/w) dextran T500, 6.2 % (w/w) polyethylene glycol 3,350, 0.33 M sucrose, 5 mM potassium phosphate (pH 7.8), and 3 mM KCl. Three successive rounds of partitioning yielded the final upper phases (U3 and U3′) and lower phase (L3). The combined upper phase was enriched with plasma membrane vesicles, and the lower phase contained intracellular membranes. Distribution of specific activity of the different membrane marker enzymes in the microsomes after two-phase partition is shown in Online Resource 1. The final upper phase was diluted fivefold in ice cold Tris–HCl dilution buffer (10 mM, pH 7.4) containing 0.33 M sucrose. The fraction was centrifuged at 140,000*g* for 60 min. The resultant pellets were then re-suspended in Tris–HCl dilution buffer and used immediately for enzyme activity analysis. All procedures were carried out at 4 °C. The activity of plasma membrane NADPH oxidase was assayed based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilideinner salt (XTT) by O_2^- radicals as described by Sagi and Fluhr (2001). Each 1 ml reaction mixture contained 100 mM Tris–HCl (pH 7.5), 2 mM $CaCl_2$, 0.5 mM XTT, 0.1 mM NADPH, and 10 μg upper phase proteins. XTT reduction was monitored at 470 nm in the presence and absence of 50 units SOD.

Superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), dehydroascorbate reductase (DHAR, EC 1.8.5.1), and glutathione reductase (GR, EC 1.6.4.2) were extracted and assayed according to the protocols of Li and Cheng (2008).

UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT, EC 2.4.1.91) was extracted as described by Li et al. (2013a). In a final assay volume of 200 μl, reaction mixture contained 100 mM buffer (Hepes–KOH, pH 8.0), 250 mM $MgCl_2$, 2 mM DTT, 9 mM UDP-galactose, 0.3 mM cyanidin, and 10 μl enzyme extract. The reaction mixture was incubated at 37 °C for 10 min and terminated by the addition of 50 μl 35 % trichloroacetic acid. The reaction mixture was extracted twice with ethylacetate, which was then evaporated completely using N_2 gas. The final reaction product (cyanidin-3-galactoside) was dissolved with 80 % methanol and analyzed using HPLC at 525 nm.

Total ascorbate and total glutathione were extracted and analyzed as described by Li and Cheng (2008). Superoxide anion and hydrogen peroxide were extracted and analyzed as described by Chen et al. (2013a).

Real-time qPCR expression analysis

Total RNA was isolated using the SDS-phenol method according to Malnoy et al. (2001). First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit (Takara, Dalian, China), according to the manufacturer's protocol. Real-Time PCR was performed with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Green MasterMix (SYBR Premix EX Taq™, Dalian, China). *MdActin* was used to standardize the cDNA samples for different genes. The primers for *MdActin*, *MdCRY2*, *MdHY5, MdCOP1, MdMYB10, MdPAL, MdCHS, MdF3H, MdDFR, MdANS, MdUFGT1*, and *MdUFGT2* are shown in Online Resource 1.

Statistical analysis

All data were statistically analyzed by *t* test and Least Significant Difference (LSD) using SPSS 16.0 (SPSS Inc. Chicago, IL, USA).

Results

Time course changes of anthocyanin synthesis after sunlight exposure

After the bagged fruits were directly exposed to sunlight, transcript levels of *MdCRY2* reached maximum levels at day 5 and then decreased (Online Resource 1). In contrast, expression levels of *MdHY5*, *MdCOP1,* and *MdF3H* peaked at day 3 and then decreased slightly (Fig. 1a, b, f). Transcript levels of *MdMYB10*, *MdCHS,* and *MdUFGT2* peaked at day 1 (Fig. 1c, e, j). *MdMYB10* expression changed slightly over the course of the exposure period, whereas that of *MdCHS* and *MdUFGT2* decreased sharply 7 days after sunlight exposure. Transcript levels of *MdPAL*, *MdDFR*, *MdANS,* and *MdUFGT1* initially increased, and then remained unchanged after sunlight exposure (Fig. 1d, g, h, i). UFGT activity and the concentration of cyanidin-3-galactoside (which accounts for more than 99 % of anthocyanin in apple peels, data not shown) all increased gradually after sunlight exposure (Fig. 1k, i). The concentrations of quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside changed in similar patterns to that of cyandin-3-galactoside, whereas that of other phenolic compounds showed little or no response to sunlight exposure (Online Resource 1). UV attenuation, after the bagged fruits were exposed to sunlight, did not affect the expression levels of *MdCRY2*, *MdHY5,* and *MdCOP1*, but significantly inhibited that of eight other genes and the activity of UFGT. The synthesis of cyanidin-3-galactoside was completely inhibited by UV attenuation (Fig. 1). For other phenolic compounds, the synthesis of quercetin-3-glycosides such as quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside were significantly inhibited. Synthesis rates of other phenolic compounds were hardly changed under UV-attenuating conditions (Online Resource 1).

Time course changes of ROS production and the antioxidant system after sunlight exposure

Concentrations of superoxide anion and hydrogen peroxide both increased after exposing bagged fruits to sunlight (Fig. 2a, b). UV attenuation lowered the concentration of superoxide anion but left hydrogen peroxide concentration unaffected. The activity of plasma membrane NADPH oxidase increased gradually and then remained unchanged at 7 days after direct sunlight exposure. However, NADPH oxidase activity did not markedly change under UV-attenuating conditions (Fig. 2c).

After sunlight exposure, SOD activity remained unchanged at first and then increased, with similar values observed with and without UV attenuation (Fig. 2d). APX activity decreased slightly and then increased, with similar values observed with and without UV attenuation (Fig. 2e). MDHAR, DHAR, and GR activities varied in similar patterns; they decreased at first and then increased, with lower values observed under UV attenuation (Fig. 2f–h). Concentrations of total ascorbate and glutathione increased gradually after sunlight exposure (Fig. 2i, j), but UV attenuation did not change the concentrations of these two metabolites.

Time course changes of chlorophyll and the xanthophyll cycle after sunlight exposure

Chlorophyll concentration decreased at day 3 and remained unchanged under direct sunlight exposure, but increased and then plateaued under UV-attenuating conditions. As a result, chlorophyll concentrations significantly increased with UV attenuation after 3 days of sunlight exposure (Fig. 3a). The xanthophyll cycle pigment pool size (V + A + Z) on a peel fresh weight basis increased gradually after sunlight exposure, with similar values observed with and without UV attenuation (Fig. 3b). However, pool size on a chlorophyll basis changed in different patterns (Fig. 3c), increasing under direct sunlight exposure but decreasing with UV attenuation at day 3. As a result, the chlorophyll-based pool size was significantly lower under UV-attenuating conditions. De-epoxidation of xanthophyll cycle pigments increased sharply at day 1 and then increased slowly during the following days (Fig. 3d). However, de-epoxidation was higher under UV-attenuating conditions after 3 days of sunlight exposure.

Hydrogen peroxide treatments

Exogenous hydrogen peroxide treatment did not change the expression levels of *MdMYB10*, *MdPAL*, *MdCHS*, *MdF3H*, *MdDFR*, *MdANS*, *MdUFGT1* and *MdUFGT2*, UFGT activity, or the concentrations of cyandin-3-galactoside and other phenolic compounds before sunlight exposure treatment (Fig. 4, Online Resource 1). However, 3 days after direct sunlight exposure, levels of *MdMYB10*, *MdPAL*, *MdCHS*, *MdDFR,* and *MdANS* increased gradually and then plateaued with increasing hydrogen peroxide concentrations. Meanwhile, levels of *MdF3H* and *MdUFGT1* expression, UFGT activity, and cyanidin-3-galactoside concentration remained unchanged when the concentration of hydrogen peroxide was less than 10 mM. However, all of these parameters increased markedly with further increase in hydrogen peroxide concentration. Levels of *MdUFGT2* were not affected by exogenous hydrogen peroxide treatment (Fig. 4). Concentrations of quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside changed in similar patterns to that of cyanidin-3-

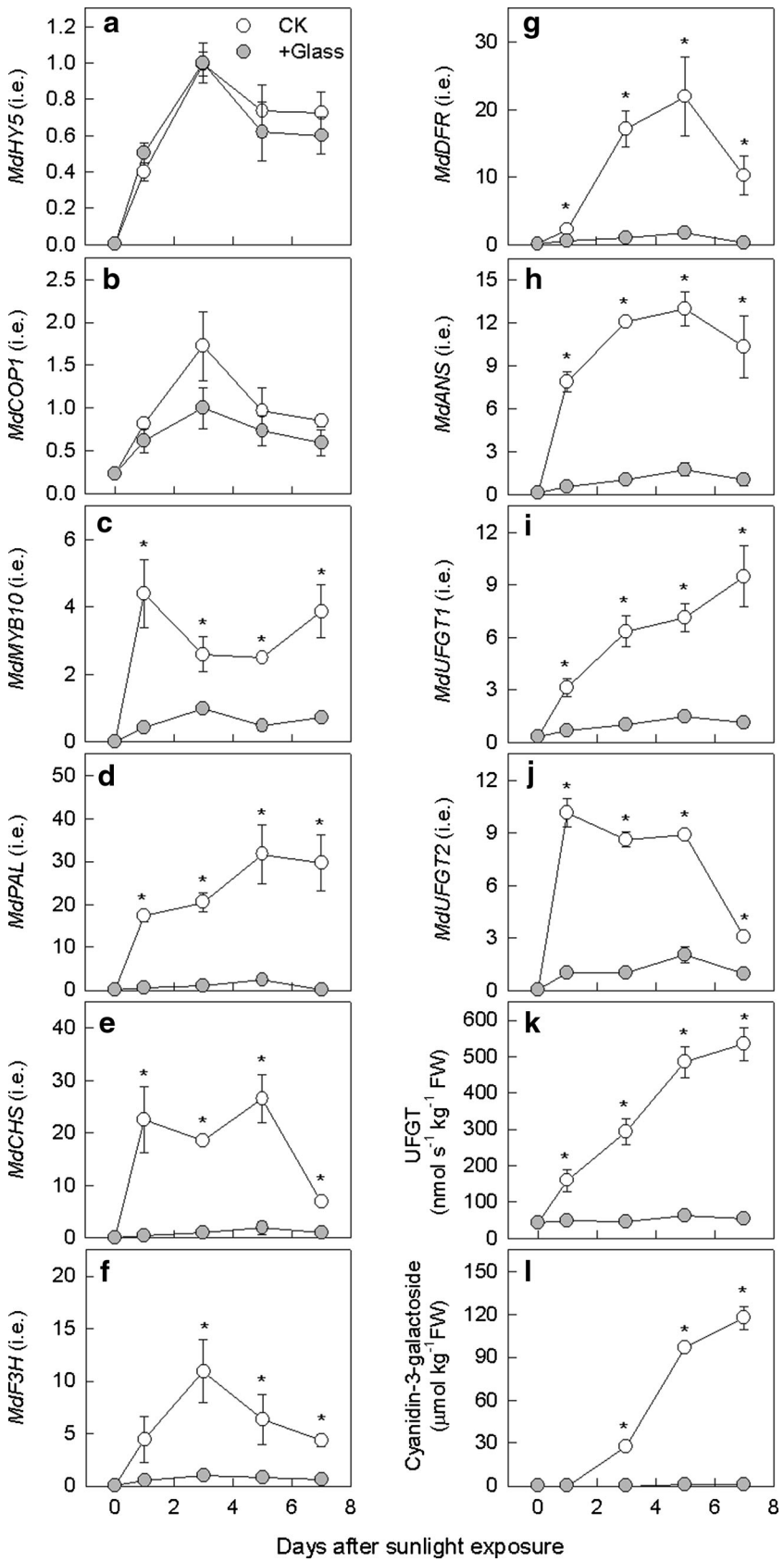

Fig. 1 Transcription levels of *MdHY5* (**a**), *MdCOP1* (**b**), *MdMYB10* (**c**), *MdPAL* (**d**), *MdCHS* (**e**), *MdF3H* (**f**), *MdDFR* (**g**), *MdANS* (**h**), *MdUFGT1* (**i**) and *MdUFGT2* (**j**), activities of UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT, **k**), and concentrations of anthocyanin (**l**) in 'Golden Delicious' apple fruit peels after directly exposing bagged fruits to sunlight (CK) or with UV attenuation by 3-mm glass (+Glass). The *asterisk* indicates a significant difference between "CK" and "+Glass" at $P < 0.05$, *t* test. Each data point represents mean ± SE ($n = 5$)

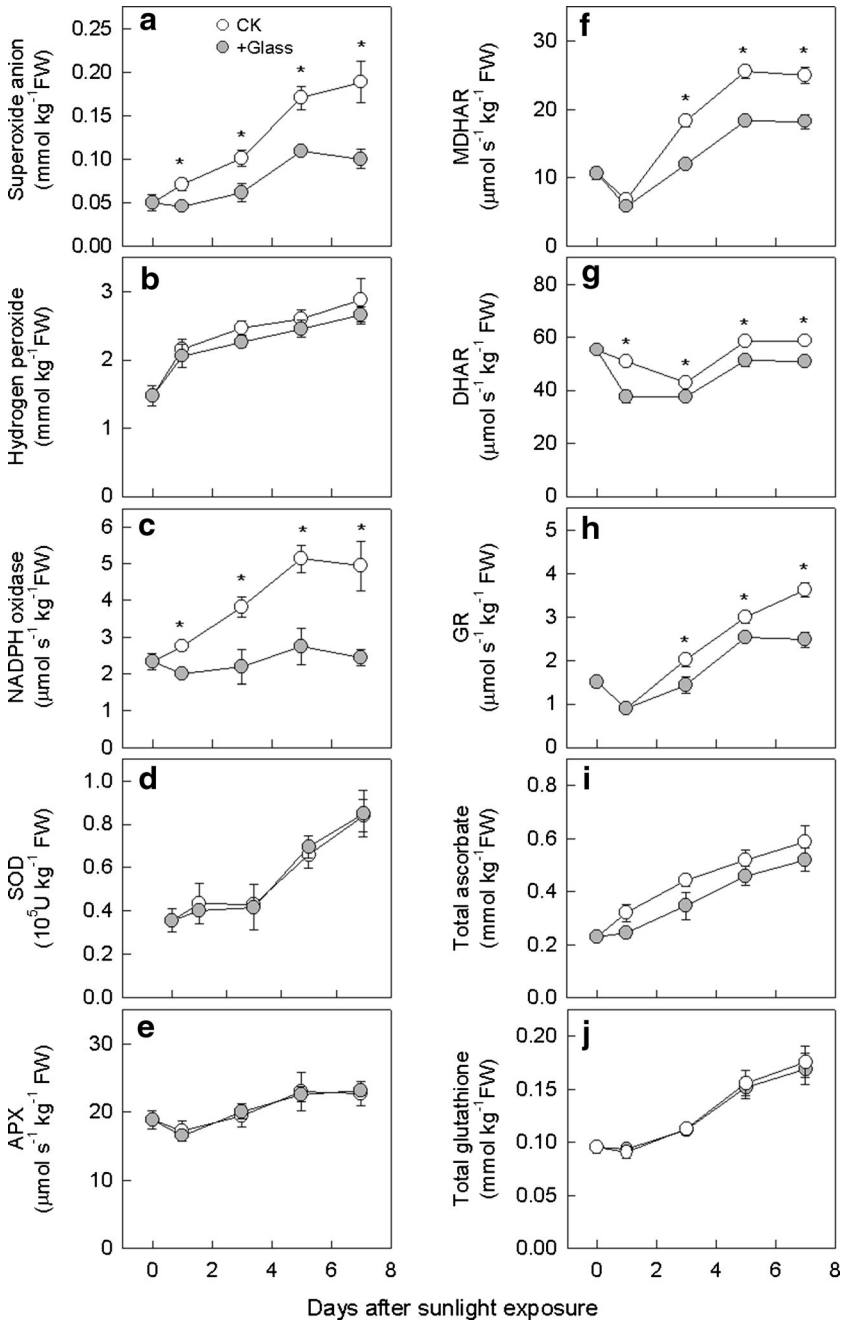

Fig. 2 Concentrations of superoxide anion (**a**) and hydrogen peroxide (**b**), activities of plasma membrane NADPH oxidase (**c**), superoxide dismutase (SOD, **d**), ascorbate peroxidase (APX, **e**), monodehydroascorbate reductase (MDHAR, **f**), dehydroascorbate reductase (DHAR, **g**) and glutathione reductase (GR, **h**), and concentrations of total ascorbate (**i**) and glutathione (**j**) in 'Golden Delicious' apple fruit peels after directly exposing bagged fruits to sunlight (CK) or with UV attenuation by 3-mm glass (+Glass). The *asterisk* indicates a significant difference between "CK" and "+Glass" at $P < 0.05$, t test. Each data point represents mean ± SE ($n = 5$)

galactoside (Online Resource 1). Concentrations of chlorogenic acid and procyanidin B2 remained unchanged until the exogenous hydrogen peroxide concentration increased to 50 mM, whereas that of other phenolic compounds did not significantly change with hydrogen peroxide treatment. Under UV-attenuating conditions, the expression levels of these eight genes, UFGT activity, and the concentrations of cyandin-3-galactoside, quercetin-3-galactoside, quercetin-3-glucoside and quercetin-3-rutinoside were significantly lower compared to that without UV attenuation. The concentration of other phenolic compounds showed slight or no variation with and without UV attenuation.

DPI treatments

Before and after sunlight exposure treatments, DPI significantly inhibited plasma membrane NADPH oxidase activity in fruit peels (Fig. 5a). When studying ROS production, DPI treatment did not affect the concentrations of superoxide anion and hydrogen peroxide before sunlight exposure (Fig. 5b, c). However, 5 days after sunlight exposure, concentrations of superoxide anion and hydrogen peroxide in DPI-treated fruit peels were significantly higher under direct sunlight exposure, but significantly lower under UV-attenuating conditions.

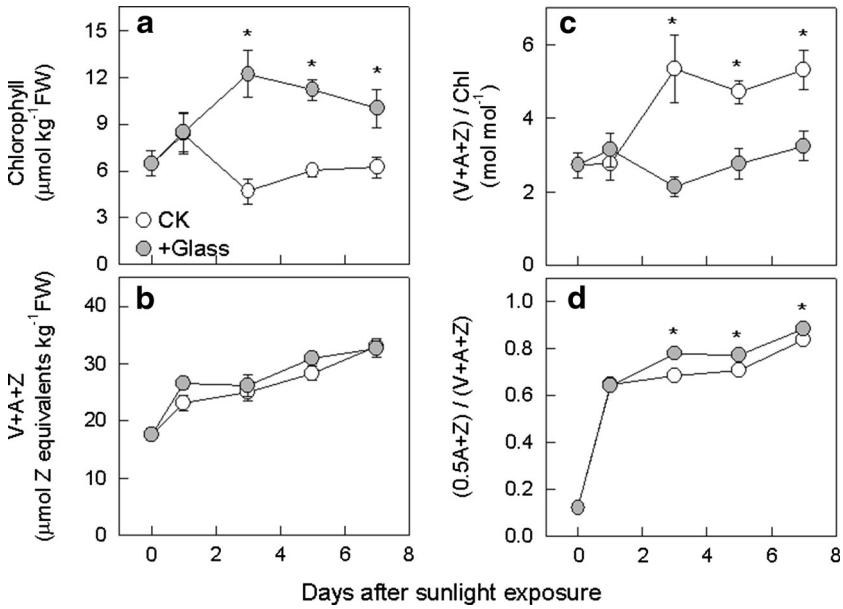

Fig. 3 Concentrations of chlorophyll (**a**), the xanthophyll cycle pool size (V + A + Z) on the basis of peel fresh weight (**b**) or chlorophyll content (**c**), and the de-epoxidation of xanthophyll pigments [(0.5A + Z)/(V + A + Z), **d**] in 'Golden Delicious' apple fruit peels after directly exposing bagged fruits to sunlight (CK) or with UV attenuation by 3-mm glass (+Glass). The *asterisk* indicates a significant difference between "CK" and "+Glass" at $P < 0.05$, *t* test. Each data point represents mean ± SE ($n = 5$)

DPI treatment did not affect the gene expression levels, UFGT activity, or concentrations of cyanidin-3-galactoside and other phenolic compounds before sunlight exposure (Fig. 6, Online Resource 1). Levels of *MdMYB10* and *MdUFGT1* were significantly lower, whereas that of *MdPAL*, *MdCHS*, *MdF3H,* and *MdANS* did not change in DPI-treated fruit peels under direct sunlight exposure. Lower DPI concentration (10 μM) did not change the levels of *MdDFR* and *MdUFGT2*, whereas the higher concentration treatment (50 μM) slightly lowered their levels under direct sunlight exposure (Fig. 6e, h). The expression levels of all eight genes were similar in peels with and without DPI treatment under UV-attenuating conditions. The activity of UFGT and the concentrations of cyanidin-3-galactoside, quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rutinoside changed in similar patterns to that of *MdMYB10* and *MdUFGT1*. Concentrations of other phenolic compounds were not changed by DPI treatments.

Discussion

After directly exposing bagged 'Golden Delicious' apple fruits to sunlight, this traditional non-red apple cultivar synthesized abundant levels of anthocyanin in fruit peels (Fig. 1, Online Resource 1), consistent with our previous studies (Chen et al. 2013a; Bi et al. 2014). Solar UV-A and UV-B attenuation with 3-mm glass significantly down-regulated the expression levels of *MdMYB10* and downstream structural genes (*MdPAL*, *MdCHS*, *MdF3H*, *MdDFR*, *MdANS,* and *MdUFGT1*) as well as the activity of UFGT, and completely inhibited anthocyanin synthesis (Fig. 1). These results indicate that the synthesis of anthocyanin in apple peel was regulated by UV irradiation alone, instead the visible sunlight.

In plants, UV-A and UV-B light photoreceptors have been identified and demonstrated to be involved in UV signal transduction pathways (Brown and Jenkins 2008; Favory et al. 2009; Jenkins 2009; Li et al. 2012, 2013b; Jaakola 2013; Peng et al. 2013). During this study, expression levels of *MdHY5*, *MdCOP1,* and *MdCRY2* were not affected by UV attenuation, suggesting that non-specific UV signaling regulated MYB expression and anthocyanin synthesis in apple peel. Hideg et al. (2012) and Wargent and Jordan (2013) suggested that UV-B-specific perception and UVR8/COP1/HY5 signaling pathways comprise the main regulatory mechanism under narrow-band, low UV-B conditions, whereas ROS-mediated signaling, DNA damage response signals, phytohormones, and MAPK signaling appear to be restricted to broad-band, high UV-B conditions. In the present study, bagged fruits used were grown in dark conditions prior to sunlight exposure, indicating that solar UV-B irradiation might have a relatively strong effect on the fruits.

Our previous studies indicate that ROS might be involved in the regulation of anthocyanin synthesis in apple peel after exposing bagged fruits to natural light conditions (Chen et al. 2013a; Bi et al. 2014). Along with the inhibition of anthocyanin synthesis, UV attenuation did not change the activities of SOD and APX (Fig. 2), the two key enzymes used for ROS scavenging in apple peel (Li and Cheng 2008). However, UV attenuation significantly lowered the accumulation of superoxide anion (Fig. 2), indicating that UV light might regulate anthocyanin synthesis through controlling ROS production. Indeed, the fact that

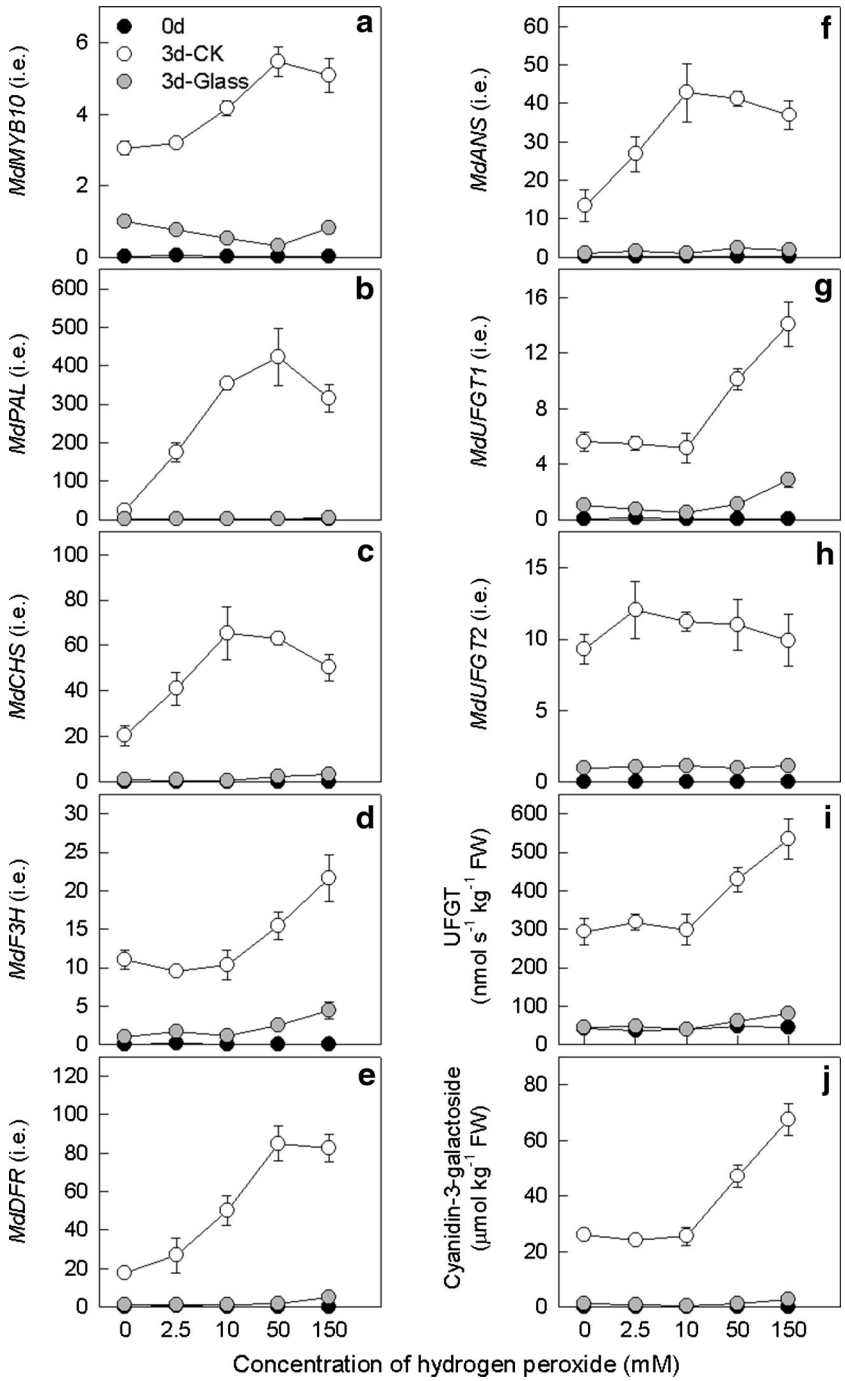

Fig. 4 Transcription levels of *MdMYB10* (**a**), *MdPAL* (**b**), *MdCHS* (**c**), *MdF3H* (**d**), *MdDFR* (**e**), *MdANS* (**f**), *MdUFGT1* (**g**) and *MdUFGT2* (**h**), activities of UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT, **i**), and concentrations of anthocyanin (**j**) in 'Golden Delicious' apple fruit peels after bagged fruits were treated with different concentrations of hydrogen peroxide and directly exposed to sunlight (CK) or with UV attenuation by 3-mm glass (Glass) for 0 or 3 days. Each data point represents mean ± SE ($n = 5$)

exogenous application of hydrogen peroxide enhanced anthocyanin synthesis (Fig. 4) demonstrates that the accumulation of ROS could up-regulate anthocyanin synthesis in fruit peel. Compared with direct sunlight irradiation, the reduction of anthocyanin synthesis in hydrogen peroxide-treated peels under UV-attenuating conditions indicates that endogenous ROS production still plays a key role in regulating anthocyanin synthesis in apple peels.

Under high-light conditions (Li and Cheng 2008; Li et al. 2008) or conditions where UV-B impairs photosynthetic electron transport (Jansen et al. 1998), the production of ROS was shown to increase in the chloroplast of fruit peel (Jenkins 2009). Meanwhile, UV-B light was shown to stimulate plasma membrane NADPH oxidase and then increase ROS production in the extracellular space (Fig. 2; Tossi et al. 2009, 2011). In the present study, inhibition of NADPH oxidase activities by the unique inhibitor DPI markedly down-regulated anthocyanin synthesis in apple peel under direct sunlight irradiation conditions (Figs. 5, 6), suggesting that the ROS produced via

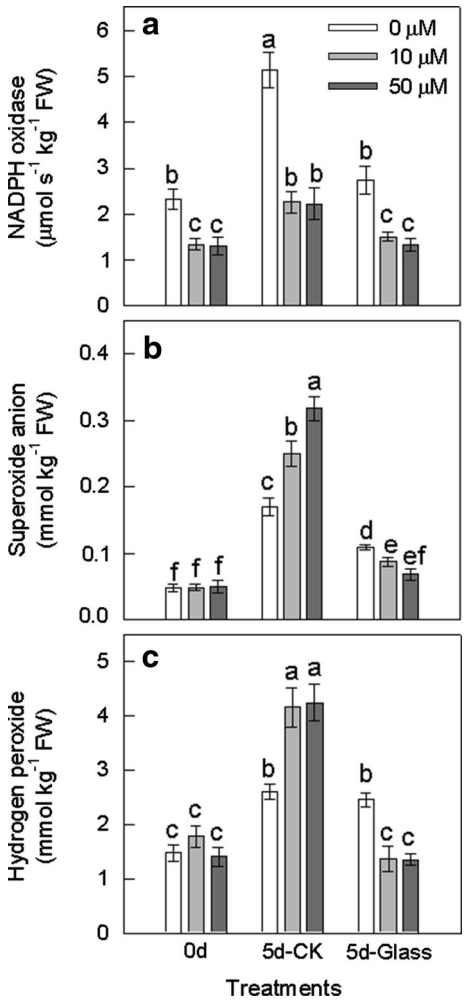

Fig. 5 Activities of plasma membrane NADPH oxidase (**a**) and concentrations of superoxide anion (**b**) and hydrogen peroxide (**c**) in 'Golden Delicious' apple fruit peels after bagged fruits were treated with different concentrations of diphenyleneiodonium chloride (DPI) and directly exposed to sunlight (CK) or with UV attenuation by 3-mm glass (Glass) for 0 or 5 days. *Different letters above the bars* indicate significant differences at $P < 0.05$, least significant difference (LSD). Each data point represents mean ± SE ($n = 5$)

plasma membrane NADPH oxidase controlled anthocyanin synthesis in apple peel. The similar patterns of change in NADPH oxidase activity and anthocyanin concentration in fruit peels also support this hypothesis (Figs. 1, 2).

The xanthophyll cycle, in which V is converted to Z via the intermediate A under light conditions, can thermally dissipate excess excitation energy before such energy triggers the formation of ROS in chloroplasts (Demmig-Adams and Adams 1992). Under high light, the xanthophyll cycle usually operates with high efficiency in fruit peels (Cheng and Ma 2004; Li and Cheng 2008; Li et al. 2008). Compared to UV attenuation, the relatively higher observed xanthophyll cycle pool size on a chlorophyll basis combined with lower de-epoxidation under direct sunlight irradiation (Fig. 3) suggests that excess excitation energy was relatively lower under this condition. Correspondingly, ROS levels produced in chloroplast should be lower. This indicates that ROS produced in chloroplast was not a major contributor to anthocyanin synthesis regulation. Under UV-attenuating conditions it was shown that DPI treatment could inhibit plasma membrane NADPH oxidase and the subsequent production of ROS through this mechanism (Fig. 5). Since plasma membrane NADPH oxidase activity was already too low to produce enough ROS to trigger anthocyanin synthesis under UV-attenuating conditions (Fig. 2), the further observed decrease in ROS production by DPI treatment did not significantly change the rate of anthocyanin synthesis (Fig. 6). Under direct sunlight irradiation, increased ROS concentrations in DPI-treated fruit peels (Fig. 5) should come from other processes. This further demonstrates that with the exception of the plasma membrane NADPH oxidase pathway, other ROS production processes contributed little to the regulation of anthocyanin synthesis. As anthocyanin may lower ROS production by light attenuation (Smillie and Hetherington 1999; Merzlyak et al. 2008), it is reasonable to surmise that reduction of anthocyanin in DPI-treated fruit peels may increase ROS production via other processes such as chloroplastic photosynthetic electron transport (Li et al. 2008). Recently, it was found that the anthocyanin synthesis pathway was redox sensitive, and ascorbate was involved in the regulation of anthocyanin synthesis in *Arabidopsis thaliana* under high light (Page et al. 2012). This may explain how activities of enzymes associated with the ascorbate–glutathione cycle became faster under direct sunlight conditions, compared to that with UV attenuation (Fig. 2).

In addition, analysis of the apple genome (Velasco et al. 2010) revealed the existence of two *MdUFGT* genes (Online Resource 1). It was clear that the synthesis of anthocyanin was related to *MdUFGT1*, and not *MdUFGT2* (Figs. 1, 4, 6), indicating that the two *MdUFGT*s play different roles in plants. The regulation of anthocyanin synthesis by *MdMYB10* should occur through control of *MdUFGT1* instead of other up-stream structural genes involved in anthocyanin synthesis. This was supported by the fact that the major difference in phenolic compounds under the three treatments was in the terms of anthocyanin and quercetin-3-glycoside (Online Resource 1), although the expression levels of other structural genes (*MdPAL, MdCHS, MdF3H, MdDFR* and *MdANS*) were also enhanced by UV irradiation and H_2O_2 treatment (Figs. 1, 4). Given the fact that MYB10 up-regulated the whole phenolic pathway in apple peels, the synthesis of most phenolic compounds should be enhanced. More importantly, of the structural genes in the anthocyanin synthesis pathway, it was shown that only *MdUFGT1* responded to

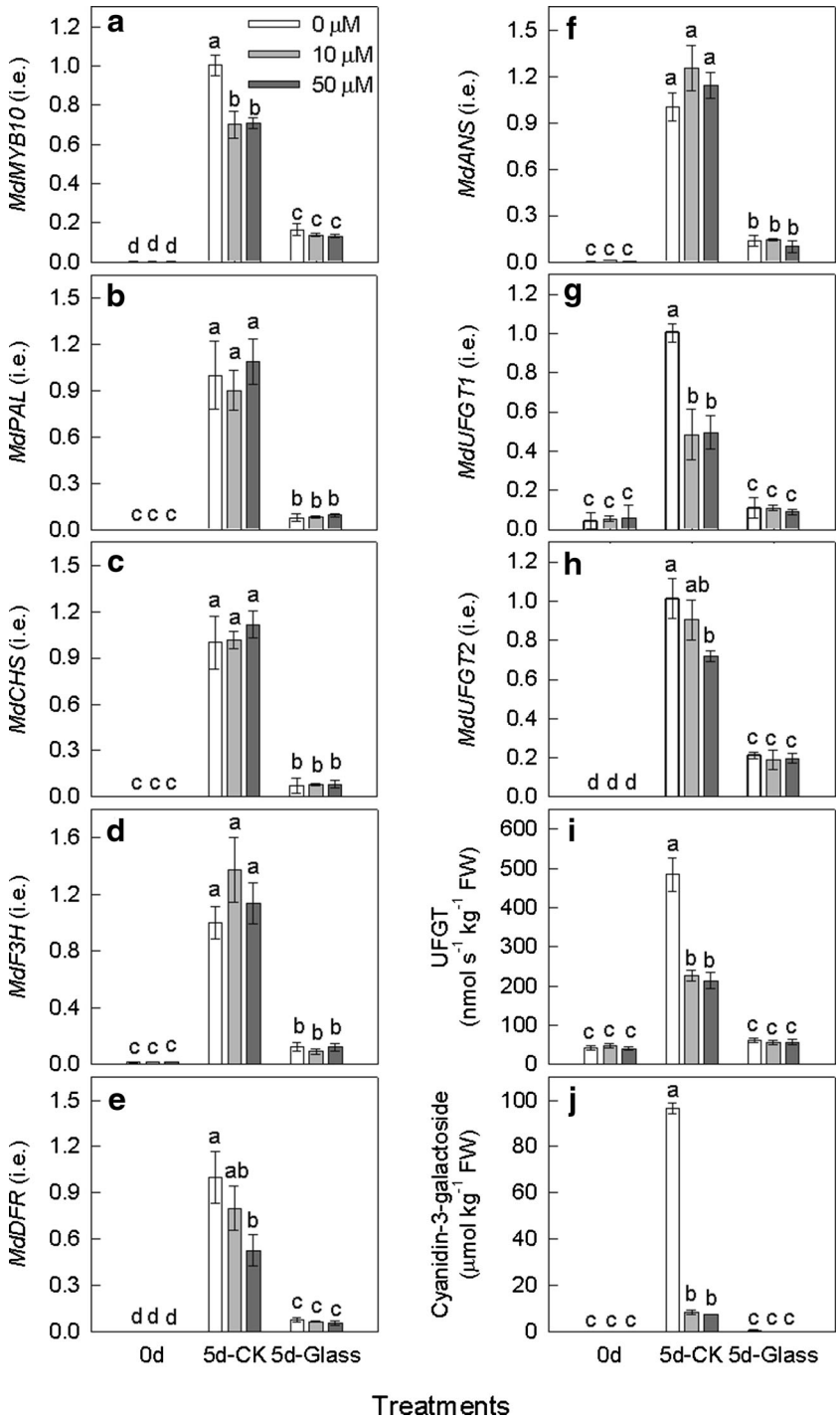

Fig. 6 Transcription levels of *MdMYB10* (**a**), *MdPAL* (**b**), *MdCHS* (**c**), *MdF3H* (**d**), *MdDFR* (**e**), *MdANS* (**f**), *MdUFGT1* (**g**) and *MdUFGT2* (**h**), activities of UDP-glycose:flavonoid 3-O-glycosyltransferase (UFGT, **i**), and concentrations of anthocyanin (**j**) in 'Golden Delicious' apple fruit peels after bagged fruits were treated with different concentrations of diphenyleneiodonium chloride (DPI) and directly exposed to sunlight (CK) or with UV attenuation by 3-mm glass (Glass) for 0 or 5 days. *Different letters above the bars* indicate significant differences at $P < 0.05$, least significant difference (LSD). Each data point represents mean ± SE ($n = 5$)

DPI treatment in similar patterns to *MdMYB10* and anthocyanin concentrations (Fig. 6). The enhancement of other structural gene expression levels by UV or H_2O_2 might be due to the enhanced synthesis of cyanidin-3-galactoside and quercetin-3-glycoside. However, it should be noted that the enhanced expression of these genes may also contribute to the synthesis of these compounds in apple peel.

In general, solar UV irradiation regulated anthocyanin synthesis in apple peel. This regulation occurred through control of the production of ROS via plasma membrane NADPH oxidase. MdMYB10 regulated anthocyanin synthesis through *MdUFGT1* in apple peel.

Author Contribution PL conceived and designed research. JZ, CC and DZ conducted experiments. JZ, CC

and HL analyzed data. JZ wrote the manuscript. PL and FM revised the manuscript. All authors read and approved the manuscript.

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