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Accumulation of flavanols and expression of leucoanthocyanidin reductase induced by postharvest UV-C irradiation in grape berry

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ABSTRACT. To examine the effect of postharvest ultraviolet C (UV-C) irradiation on flavanol polyphenol accumulation in the grape berry, we investigated total flavanol polyphenol content, the enzyme activity of leucoanthocyanidin reductase (LAR), and transcription of *Vv lar*1 and *Vv lar*2 using spectrophotometry, real-time polymerase chain reaction, and western blot analysis in 5-year-old *Vitis vinifera* L. cv. Cabernet Sauvignon plants. Our results indicated that the accumulation of flavanol polyphenol reached its highest value when exposed to UV-C irradiation for 30 min. Additionally, UV-C irradiation induced the transcription of *Vv lar*1 and *Vv lar*2 and the synthesis of LAR1 and LAR2 proteins, resulting in increased accumulation of flavanol polyphenol in the grape berry. Moreover, these effects were associated with the length of time of UV-C irradiation.

Key words: Flavanol polyphenol content; Ultraviolet C; Leucoanthocyanidin reductase; *Vitis vinifera*

INTRODUCTION

Grapes (*Vitis vinifera*) are among the most widely consumed fruits, and the demand for grapes and grape products is increasing, in part because of their associated health benefits (Capanoglu et al., 2013). This increase may be attributed to the presence of antioxidants, particularly phenolic compounds in grapes and red wine (Girona et al., 2009; Schroeter et al., 2010). Flavanol polyphenol content is not only an important feature in grape berry, but also gives many sensory qualities to grape wine, such as color, flavor, clarity, convergence, and browning (Girona et al., 2009; Liu et al., 2011, 2012). Therefore, the quality of grape fruit and wine can be improved by regulating the flavanol polyphenol content.

The biosynthesis and accumulation of polyphenols are regulated by environmental conditions such as temperature (Wen et al., 2008; Crifò et al., 2011), illumination (Wang et al., 2010, 2012; Koyama et al., 2012), and water (Acevedo-Opazo et al., 2010; Ollé et al., 2011; Quiroga et al., 2012). Numerous studies have reported that ultraviolet (UV) irradiation induces the accumulation of polyphenols (Wang et al., 2010; Guerrero et al., 2010; Interdonato et al., 2011; Sergio et al., 2012; Zhang et al., 2012). Furthermore, Liu et al. (2010) showed that UV-C irradiation for 20 min was most efficient for promoting the accumulation of resveratrols and accumulation of stilbenes in UV-C-irradiated calli depended upon the genetic background and tissue type, with higher stilbene content in 2 interspecific root stocks and leaf or exocarp explants. However, both the effect of postharvest UV-C irradiation on flavanol polyphenol accumulation and the function of flavanol polyphenol, particularly that of the key enzyme-leucoanthocyanidin reductase (LAR) in grape berry, were unknown. Maugé et al. (2010) confirmed that the expression of *Vv lar* affects the biosynthesis and accumulation of flavanol polyphenols in the grape berry. Thus, we examined the effect of postharvest UV-C irradiation on flavanol polyphenol accumulation in grape berry by measuring total flavanol polyphenol content, LAR activity, and *Vv lar*1 and *Vv lar*2 transcription in 5-year-old *V. vinifera* L. cv. Cabernet Sauvignon by using spectrophotometry, real-time polymerase chain reaction (PCR), and western blot analysis.

MATERIAL AND METHODS

Plant materials

All grape plants (*V. vinifera* L. cv. Cabernet Sauvignon) were grown in a vineyard at Shanxi Agricultural University, located in Taigu County, Shanxi Province, China. Five-year-old plants were used and trained to grow on a trellis spaced at 1.0 x 2.5 m. Grape berries were hand-harvested when ripe.

UV-C irradiation

Two low-pressure, mercury-vapor, discharge lamps (ZSZ, Tanjin Guangze Special Light Source Co., Ltd., Tianjin, China) emitting quasi-monochromatic UV-C irradiation at 254 nm were used. The lamps were mounted 3.0 cm apart on a cast-iron frame with stainless steel reflector, and the UV-C lamp suspension frame was placed inside a thin box enclosure (30 cm wide x 120 cm long). Fruits were placed on a tray approximately 0.5 m from the lamp surface and exposed to a UV-C dose of 100 μW/cm, measured using a UVX radiometer (UVP, Inc.,

San Gabriel, CA, USA). Fruits were individually rotated to ensure that all sides of the fruit were exposed to UV-C. UV-treated berries and their controls were stored at 24°C under high (95%) relative humidity in plastic containers. The samples were collected at 0, 1, 5, 10, 30, and 60 min after treatment and stored at -80°C, from 10 berries randomly selected from each treatment, with 3 fruits per replicate.

Content of total phenolic, polyphenol flavanols, and proanthocyanidins

Total phenolic content was determined using the Folin-Ciocalteu reagent method (Tian et al., 2006) and reported as mg gallic acid/g fresh weight (FW). Flavanol polyphenol content was measured using the vanillin-hydrochloric acid method as described by Waterhouse et al. (2000) and the results were reported as mg catechin/g FW. Proanthocyanidin content was measured using the butanol-hydrochloric acid method as described in our previous study (Wen et al., 2005; Wen, 2005) and reported as mg catechin/g FW.

LAR preparation and assay

LAR activity (reported as mg catechin·mg^{-1} $prot^{-1}$·h^{-1}) was determined by monitoring the conversion of dihydroquercetin to (+)-catechin following the method of Gagné et al. (2009) as follows: the assay mixture contained 10 µL 1 g/L dihydroquercetin in methanol, 10 µL 20 mM NADPH, and 110 µL 0.1 M Tris-HCl buffer, pH 7.5. The reaction was initiated by adding 70 µL crude extract and incubated at 25°C for 30 min, after which the reaction was stopped by adding 200 µL ethyl acetate with vigorous vortexing. Extraction was repeated and the ethyl acetate phases were pooled and dried under nitrogen gas. Residues were dissolved in 100 µL high-performance liquid chromatography-grade methanol for chromatographic analysis. LAR products were separated on a Beckman Ultrasphere ODS (250 x 4.6 mm, 5 µm; Beckman-Coulter, Brea, CA, USA) reversed-phase column and eluted with 5% (v/v) acetic acid in water (solvent A) and methanol (solvent B), according to the following program: 5% B from 0 to 5 min, 5 to 10% B from 5 to 10 min, 10% from 10 to 16 min, 10 to 90% B from 16 to 21 min, 90% B from 21 to 31 min, 90 to 5% B from 31 to 36 min, and 5% B up to 45 min. The flow rate was set at 1 mL/min, the detection wavelength was 280 nm, and the injection volume was 50 µL. Identification and quantification were performed using an external (+)-catechin standard (Sigma, St. Louis, MO, USA). Data are reported as means of 3 assays per extract ± SD.

Protein gel blot analysis

The protein levels of LAR1 and LAR2 were quantified by western blotting as previously described (Wen et al., 2008). Primary antibodies were provided by Prof. Qiuhong Pan (College of Food Science & Nutritional Engineering, China Agricultural University, Beijing, China). For analysis, 25 µg protein was loaded onto the gel at an antibody dilution of 1:1500 for LAR1 and LAR2. After incubation with alkaline phosphatase-labeled goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) at a 1:10,000 dilution, immunoreactive proteins were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, Little Chalfont, UK). Chemiluminescence and densitometric analysis of the immunoblots was performed using the ImageJ 1.44p software, and all proteins were quantified relative to the loading control.

Real-time quantitative PCR

Total RNA in grape berries was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time PCR amplification was conducted using the TransScript II Two-Step qRT-PCR SuperMix (Beijing Transgen Biotech Co., Ltd., Beijing, China). The primer pairs of *Vv lar*1 and *Vv lar*2 were specifically designed using the Primer 5.0 software (Primer-E Ltd., Plymouth, UK) according to published sequences (primer sequences are shown in Table 1). The *Vv ubiquitin*1 gene was used as an internal control. Reactions were run on a quantitative RT-PCR 7300 system (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. Gene expression levels were calculated using the $2^{-\Delta Ct}$ method. ΔCt = Ct (target gene) - Ct (*Vv ubiquitin*1).

Table 1. Primer sequences and PCR amplified products.

Gene		Sequence of primer (5'-3')	PCR products (bp)	Accession No.
*Vv lar*1	Sense	ACGATGTCCGAACACTGAAC	187	AJ865336
	Anti-sense	TGAACGCCGCTACTACACTC		
*Vv lar*2	Sense	TCTCGACATACATGATGATGTG	166	AJ865334
	Anti-sense	TGCAGTTTCTTTGATTGAGTTC		
*Vv ubiquitin*1	Sense	GTGGTATTATTGAGCCATCCTT	182	BN000705
	Anti-sense	AACCTCCAATCCAGTTATCTAC		

Statistical analysis

Data for all analyses were analyzed using the SAS statistical software (Version 9.2, SAS Institute, Cary, NC, USA) and subjected to analysis of variance. Results among treatments were compared using Fisher least significant differences (P = 0.05). Data are reported as means ± standard error of the mean. Each experiment was performed at least 3 times and showed similar results.

RESULTS

Effect of postharvest UV-C irradiation on flavanol polyphenol accumulation in *V. vinifera* L. cv. Cabernet Sauvignon

Flavanol polyphenol contents of Cabernet Sauvignon berry were enhanced in response to postharvest UV-C irradiation as shown in Figure 1. Moreover, the peak appeared at 30 min after UV-C irradiation and the difference was statistically significant compared with the control.

Effect of postharvest UV-C irradiation on LAR activity in *V. vinifera* L. cv. Cabernet Sauvignon

UV-C irradiation increased LAR activity at 1 min after UV-C irradiation and the difference was statistically significant compared with the control as shown in Figure 2. However, LAR activity markedly decreased by following longer exposure to UV-C greater than 30 min.

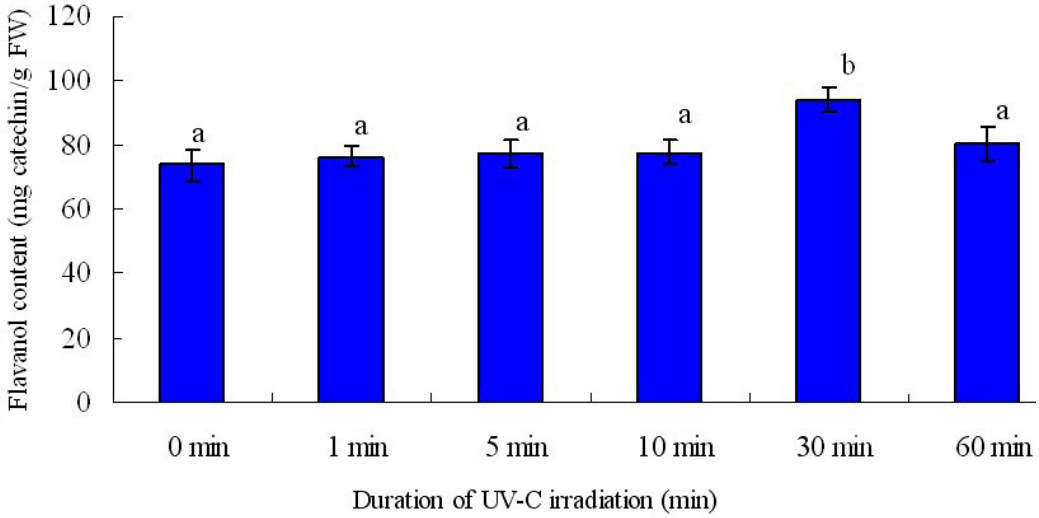

Figure 1. Effect of postharvest UV-C irradiation on flavanol polyphenol accumulation in *Vitis vinifera* L. cv. Cabernet Sauvignon. Data are reported as means ± standard deviation from three replications. Mean separation within treatments by the Fisher least significant difference at P = 0.05.

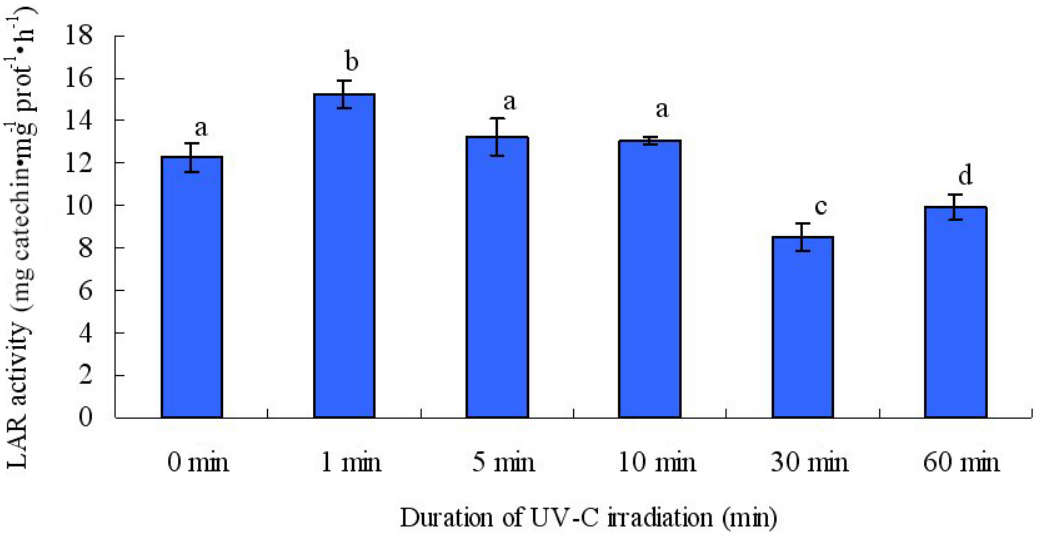

Figure 2. Effect of postharvest UV-C irradiation on leucoanthocyanidin reductase activity in *Vitis vinifera* L. cv. Cabernet Sauvignon. Data are reported as means ± standard deviation from three replications. Mean separation within treatments by the Fisher least significant difference at P = 0.05.

Effect of postharvest UV-C irradiation on LAR1 and LAR2 protein contents in *V. vinifera* L. cv. Cabernet Sauvignon

Western blot analysis demonstrated that a 43-kD polypeptide was detected in all treatments. LAR1 protein content was increased at first and then decreased. Moreover, the highest

value appeared following exposure to UV-C for 10 min (Figure 3A). However, LAR2 protein content was decreased with an increased duration of UV-C irradiation (Figure 3B).

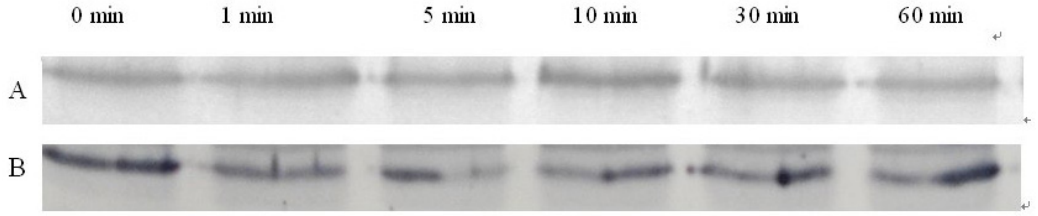

Figure 3. Effect of postharvest UV-C irradiation on LAR1 protein contents (**A**) and LAR2 protein contents (**B**) in *Vitis vinifera* L. cv. Cabernet Sauvignon. Equal protein amounts (10 µg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Thereafter, the LAR1 and LAR2 amount was immunodetected with the specific antibody.

Effect of postharvest UV-C irradiation on the expression of *Vv lar*1 and *Vv lar*2 in *V. vinifera* L. cv. Cabernet Sauvignon

As shown in Figure 4, the expression of *Vv lar*1 was lower than that of *Vv lar*2 under all treatments. Moreover, the variations of *Vv lar*1 and *Vv lar*2 in response to UV-C irradiation were the same. Compared with the control, the expression of *Vv lar*1 and *Vv lar*2 were both increased at 1, 5, and 60 min after UV-C irradiation. However, only the increase in *Vv lar*2 expression was statistically significant.

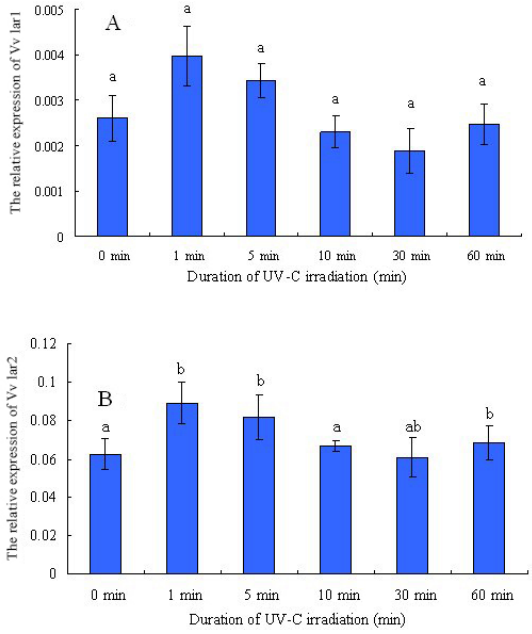

Figure 4. Effect of postharvest UV-C irradiation on the expression of *Vv lar*1 (**A**) and *Vv lar*2 (**B**) in *Vitis vinifera* L. cv. Cabernet Sauvignon. Data are reported means ± standard deviation from three replications. Mean separation within treatments by the Fisher least significant difference at P = 0.05.

DISCUSSION

UV-C irradiation induces polyphenol accumulation in many fruits, such as grape (Pan et al., 2009; Wang et al., 2010), apple (Ubi et al., 2006), pear (Kataoka and Beppu, 2004), strawberry (Higashio et al., 2005), and lemon (Interdonato et al., 2011). This leads to the accumulation of flavonoid substances (Interdonato et al., 2011; Sun et al., 2011). Consistent with previous studies, postharvest UV-C irradiation increased the total flavonoid content in *V. vinifera* L. cv. Cabernet Sauvignon in our study.

In response to elevated doses of solar radiation, an effective protective strategy is to accumulate phenolic compounds that selectively absorb UV-C irradiation in the plant cuticle and epidermis (Pontin et al., 2010; Koyama et al., 2012). These compounds also act as scavengers of reactive oxygen species in living cells to counteract the consequences of irradiation (Pontin et al., 2010; Rodrigo et al., 2011; Koyama et al., 2012). Koyama et al. (2012) found that phenolic compounds are biosynthesized through the phenylpropanoid and flavonoid pathways and originated from phenylalanine, which were among the most characterized secondary metabolic pathways in plants. Tegelberg et al. (2001) proposed that polyphenolic flavonoids and related phenols accumulate in response to UV-C irradiation. The results of our data confirmed that flavanol polyphenol contents in the Cabernet Sauvignon berry were increased significantly in response to postharvest UV-C irradiation for 30 min (Figure 1).

From leucoantocyanidin, 2,3-trans-flavan-3-ol is directly produced by LAR in grape (Maugé et al., 2010; Zhang et al., 2013). Gagné et al. (2009) showed that the highest LAR activity level was detected in pea-sized berries at 24 days after anthesis and the second activation point occurred at 67 days after anthesis, corresponding to the end of color-change in grape skins. Bogs et al. (2005) found that LAR1 expression was almost restricted to seeds, whereas LAR2 was expressed in both skin and seeds. Ollé et al. (2011) found that flavanol polyphenols accumulated in the peel, pulp, and seeds of the fruit. For this experiment, whole grape berries were sampled after UV-C irradiation at different time points. Our results indicated that LAR activity first increased and then decreased with increasing time of UV-C irradiation (Figure 2). This may be one of the reasons for flavanol polyphenol accumulation by suitable UV-C irradiation. Moreover, UV-C irradiation increased the activity of a key enzyme (phenylalanine ammonialyase, PAL) in the initial stages of the phenylpropanoid pathway and induced gene expression of chalcone synthase (Ferri et al., 2011), flavanone 3-hydroxylaase, and dihydroflavonol 4-reductase (Ubi et al., 2006). Therefore, the increased LAR activity may be related to increased substrate concentration resulting from the specific expression of structural genes in the phenylpropanoid and flavonoid pathway induced by UV-C irradiation.

Western blot analysis of the LAR1 and LAR2 proteins showed that they were affected by postharvest UV-C irradiation in grape (Figure 3). Thus, flavanol polyphenol accumulation was regulated by UV-C irradiation at the translational level. Our previous study showed that increased PAL protein content induced an increase in PAL activity during heat stress (Wen et al., 2008) and that exogenous salicylic acid-induced increase PAL protein content, leading to increased PAL activity (Wen et al., 2005; Wen, 2005). Hence, it can be concluded that increased LAR activity increased the LAR protein content, which was similar to temperature-induced or salicylic acid-induced increase in PAL activity.

Koyama et al. (2012) showed that the 2 LAR isogenes respond differently to light; *Vv*LAR1 is induced by visible light, but not in response to UV light, while *Vv*LAR2 is insensitive to light in young berry skins. However, in this study, the expression of *Vv lar*1 was lower

than that of *Vv lar*2 under all treatments. The expression of *Vv lar*1 and *Vv lar*2 were both increased at 1, 5, and 60 min after UV-C irradiation (Figure 4). This may be because these 2 LAR isogenes were previously reported to have different patterns of expression in various organs, suggesting differences in gene regulation (Bogs et al., 2005; Fujita et al., 2007). Similarly, different patterns of expression of the 2 LAR isogenes were reported during apple fruit maturation (Takos et al., 2006).

In conclusion, postharvest UV-C irradiation of grape berry may induce the transcription of *Vv lar*1 and *Vv lar*2, the synthesis of LAR, increase LAR activity, and cause accumulation of flavanol polyphenols in grape berry. Moreover, these effects were associated with the duration of UV-C irradiation.

Conflicts of interest

The authors declare no conflict of interest.

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