



Increased Cytosolic Calcium Contributes to Hydrogen-Rich Water-Promoted Anthocyanin Biosynthesis Under UV-A Irradiation in Radish Sprouts Hypocotyls

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Our previous studies showed that hydrogen-rich water (HRW) promoted the biosynthesis of anthocyanin under UV-A in radish. However, molecular mechanism involved in the regulation of the anthocyanin biosynthesis is still unclear. In this study, the role of calcium (Ca²⁺) in HRW-promoted anthocyanin biosynthesis in radish sprouts hypocotyls under UV-A was investigated. The results showed that a positive effect of HRW on the content of cytosolic calcium and anthocyanin accumulation, mimicking the effects of induced CaCl₂. Exogenous addition of Ca²⁺ chelator bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and inositol 1,4,5-trisphosphate (IP3) synthesis inhibitor neomycin partially reversed the facilitated effect of HRW. The positive effects of HRW on activity of anthocyanin biosynthetic-enzymes (*L*-phenylalanine ammonia-lyase, PAL; chalcone isomerase, CHI; dihydroflavonol 4-reductase, DFR and UDP glc-flavonoid 3-O-glucosyl transferase, UFGT) were reversed by EGTA and neomycin. Further tests confirmed that the upregulation of anthocyanin biosynthetic related genes induced by HRW was substantially inhibited by calcium antagonists. The possible involvement of CaM in HRW-regulated anthocyanin biosynthesis was also preliminarily investigated in this study. Taken together, our results indicate that IP3-dependent calcium signaling pathway might be involved in HRW-regulated anthocyanin biosynthesis under UV-A irradiation.

Keywords: calcium, hydrogen-rich water, radish sprouts, anthocyanin, UV-A

INTRODUCTION

Anthocyanins, belonging to flavonoids, are naturally occurring water-soluble pigments which confer red, orange, blue, and purple colors in fruits and vegetables (Harborne and Williams, 2000; Jaakola, 2013). In plants, anthocyanins play important physiological roles, such as scavenging free radicals, attracting insect pollination, and resisting UV radiation (Harborne and Williams, 2000; Nijveldt et al., 2001; Glover and Martin, 2012). Anthocyanins can also act as antibacterial agents against bacteria invasion (Harborne and Williams, 2000). In addition, numerous studies have shown that anthocyanins have antioxidant, anti-inflammatory, and anti-tumor-promoting effects

(Kong et al., 2003; Nam et al., 2005; Xia et al., 2006; Pascual-Teresa and Sanchez-Ballesta, 2008; Santosbuelga et al., 2014). Consuming anthocyanin-rich foods can significantly reduce the risk of chronic diseases in humans (Toufektsian et al., 2008; Abdenour and Charles, 2014; Ali et al., 2017). Therefore, the study of promoting anthocyanin biosynthesis in crops has potential to significantly impact human nutrition and health.

The biosynthesis pathway of anthocyanins is well studied in model plant *Arabidopsis* (Koes et al., 2005; Gonzalez et al., 2008; Jaakola, 2013). Anthocyanins are synthesized via the phenylpropanoid pathway and the key enzymes include phenylalaninammonio-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), and UDP-glucosyltransferase (UGT) (Gonzalez et al., 2008; Jaakola, 2013). Transcriptional control of anthocyanin biosynthesis has been studied in *Arabidopsis* toward understanding and manipulating color phenotype. WD-repeat/bHLH/MYB (MBW) complex, which was composed of TRANSPARENT TESTA GLABRA 1 (TTG1), basic-helix-loop-helix (bHLH) transcription factors, and R2R3 MYB transcription factors, plays a major regulatory role in regulating anthocyanin biosynthesis (Koes et al., 2005; Gonzalez et al., 2008; Xu et al., 2015). PRODUCTION OF ANTHOCYANIN PIGMENT (PAP) protein family of R2R3 MYB transcription factors are specifically needed for anthocyanin biosynthesis and are therefore believed to be key components of MBW complex (Borevitz et al., 2000; Shi and Xie, 2010). The biosynthesis of anthocyanins in plants is regulated by both developmental signals and environmental signals, such as plant hormones, UV, high light intensity, cold/drought stress, and nutrients deficiency stress (Jaakola, 2013; Su et al., 2014; Lotkowska et al., 2015; Xie et al., 2017). The accumulation of anthocyanin is often considered to be associated with stress defense (Xu et al., 2017).

Hydrogen gas (H_2) is a colorless and odorless inert gas. Ohsawa et al. (2007) found that H_2 could be used as a potential medical gas to selectively scavenge intracellular hydroxyl radicals and peroxynitrite in rats. Furthermore, H_2 aqueous solution—hydrogen-rich water (HRW) has been used in the clinically treatment of colon inflammation, ischemic brain injury, and diabetes, etc. (Kajiyama et al., 2009; Kamimura et al., 2011; Han et al., 2015). Studies in plants also showed that H_2 has a number of biological effects. For instance, H_2 has been reported to alleviate paraquat-induced oxidant stress in alfalfa, high salt stress in *Arabidopsis*, cadmium stress in Chinese cabbage and high light intensity stress in maize (Xie et al., 2012; Jin et al., 2013; Wu et al., 2015; Zhang et al., 2015). Previous studies also reported that H_2 could regulate cucumber adventitious root formation (Zhu et al., 2016) and prolong the shelf life in kiwifruit (Hu et al., 2018). In our previous study, we found that the application of HRW under

white light, which is the normal growth condition for radish sprouts, does not stimulate anthocyanin accumulation. However, the application of HRW under UV-A could significantly increase anthocyanin accumulation in radish sprouts hypocotyls (Su et al., 2014). However, the underlying molecular mechanisms, especially the downstream signal molecules involved in HRW-regulated anthocyanin accumulation under UV-A remain to be elucidated.

Calcium (Ca^{2+}) is an essential nutrient element for plant growth and development. In addition, Ca^{2+} is a ubiquitous secondary-messenger molecule, which participates in the signal transduction in plant development and plant resistance to both biotic and abiotic stresses (Kader and Lindberg, 2010; Schulz et al., 2013). Furthermore, it has been reported that the expression of *DFR* gene, which is a key gene involved in anthocyanin biosynthesis, was induced by Ca^{2+} (Gollop et al., 2002). Several studies have also demonstrated that Ca^{2+} stimulates the accumulation of anthocyanin (Li et al., 2004; Shin et al., 2013; Xu et al., 2014). The 1,4,5-trisphosphate (IP3) receptors (IP3Rs), located in endoplasmic reticulum membrane or tonoplast, play key roles in the regulation of Ca^{2+} signals. Binding of IP3 to the IP3Rs causes the calcium channel to open, and thus calcium ions could flow from the calcium stores to the cytoplasm (Handy et al., 2017). IP3 was shown to mediate ABA-induced synthesis of isoflavones in soybean sprouts under UV-B irradiation (Jiao et al., 2016). Studies have also shown that CaM can regulate the accumulation of anthocyanins in *Daucus carota* and grape fruits (Sudha and Ravishankar, 2003; Peng et al., 2016). The activity of CaM in the *Alternanthera bettzickiana* L. seedlings has a positive correlation with anthocyanin content (Wang et al., 2005). However, the function of Ca^{2+} in HRW-regulated anabolism of anthocyanin in radish sprouts exposed to UV-A is still unknown.

In this study, we observed an increment in cytoplasm Ca^{2+} concentration in radish protoplast when HRW was applied, which mimicked the effects of $CaCl_2$. Pharmacological and molecular experiments suggested that Ca^{2+} could act as a downstream signal molecule in HRW-promoted anthocyanin accumulation under UV-A. These results are expected to further elucidate the mechanism of HRW-regulated anthocyanin biosynthesis. Furthermore, this study could also extend our knowledge of functional mechanism of H_2 in higher plants and provide a theoretical basis and guidance for the production of anthocyanin-rich radish sprouts in greenhouse and environment-controlled facilities.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Radish (*Raphanus sativus* L.) seeds were sterilized by 0.7% NaClO and soaked in deionized water at 25°C for 6 h, and then uniform seeds were selected and spread evenly in growth trays and cultured in a dark incubator at 25°C. After incubation for 36 h, sprouts were transferred into an environment-controlled chamber and exposed to UV-A irradiation (central wavelength 365 nm; the employed UV-A intensity was 5.5 W m⁻²). At the same time, sprouts were incubated in different treatment

Abbreviations: EGTA, bis (β -aminoethylether)-N,N,N',N'-tetraacetic acid; IP3, inositol 1,4,5-trisphosphate; HRW, hydrogen-rich water; CaM, calmodulin; RR, ruthenium red; W7, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide; W5, N-(6-Aminohexyl)-1-naphthalenesulfonamide Hydrochloride; TFP, trifluoperazine; Fluo-3/AM, Fluo-3 acetoxyethyl ester.

solutions as described in the corresponding figure legends. The sample without chemicals was the control (Con). The treatment solutions were replaced every 12 h with new prepared solution. The relative humidity and temperature was 80% and 25°C, respectively. After various treatments, sprouts were photographed and hypocotyl tissues were used immediately or frozen with liquid nitrogen and stored at -80°C for further use.

The Preparation of HRW

Purified H₂ gas (99.99%, v/v) generated from a hydrogen gas generator (SHC-300, Saikesaisi Hydrogen Energy Co., Ltd, Jinan, China) was bubbled into 1,000 mL distilled water (pH 5.87, 25°C) at a rate of 150 mL min⁻¹ for 60 min. In our experimental conditions, this is a sufficient time to saturate the solution with H₂. The saturation of H₂ in water was 781 μmol L⁻¹ at 25°C. The H₂ concentration in freshly prepared HRW (100% saturation) analyzed by a needle-type Hydrogen Sensor (Unisense) was 830 μmol L⁻¹. After 12 h, the concentration of H₂ in HRW still maintained about 150 μmol L⁻¹, which was much higher than 0.3 μmol L⁻¹ in sprouts in normal condition (see Figure S1).

Measurement of Anthocyanin Content

Anthocyanin content was determined according to the method of Xie et al. (2016) with slight modifications. Briefly, 0.5 g of hypocotyl samples were soaked in 5 mL 1% HCl-methanol solution and incubated for 12 h at 4°C. The absorbancies of the extracts at 530 and 675 nm were measured by spectrophotometrical meter. And (A₅₃₀-0.25 × A₆₇₅) per gram fresh weight was used to quantify the relative amount of anthocyanin.

Measurement of Cytosolic Calcium Concentration

The radish mesophyll protoplasts were isolated according to the methods of Yoo et al. (2007) and Hagimori and Nagaoka (1992) with minor modifications. 0.5 mm leaf strips were cut from the middle part of the leaves and transferred to the enzyme solution [0.5 M MES (pH 7.5) containing 1.5% (w/v) cellulase R10, 0.4% macerozyme R10, 0.4 mM mannitol, 20 mM KCl, 10 mM CaCl₂, and 0.1% BSA] immediately. After vacuum infiltrated in the dark for 30 min at 25°C, the leaf strips were transferred to an incubator to continue the digestion in the dark for 4 h at room temperature. Then the enzyme solution was washed twice with W5 solution. The protoplasts were collected after centrifugation and re-suspended in MMG [4 mM 4-morpholineethanesulfonic acid (MES, pH 5.7) containing 0.4 M mannitol and 15 mM MgCl₂] solution. Protoplasts were kept at room temperature.

Cytosolic calcium concentration was measured by the calcium fluorescent probe Fluo-3/AM (Molecular Probes) based on the method of Yan et al. (2015) and Zhang et al. (1998). Briefly, 1 mM Fluo-3/AM in anhydrous DMSO (5 μL) was added to radish mesophyll protoplasts. After incubation at 4°C in the dark for 2 h, the protoplasts were washed three times with isotonic solution to wash away the residual dye. Then the protoplasts were incubated with incubation solution [containing 20 mM Fluo-3/AM, 0.5 M mannitol, 4 mM MES (pH 5.7), and 20 mM KCl] at 25°C for 1 h. The fluorescence of Ca²⁺ was measured after various reagents (as

described in **Figure 2**) were added. Images of 25 protoplasts for each treatment in three independent experiments were observed using a PE (Ultra View VOX) laser scanning confocal microscope (LSCM) with the 488 nm excitation wavelength. The emission fluorescence was filtered by a 515 nm filter to eliminate the autofluorescence of chlorophyll. The fluorescence of protoplasts on acquired images were analyzed by image J software (NIH). Data were calculated as means ± SE of pixel intensities.

Anthocyanin Profiles Analyzed by Ultra-Performance Liquid Chromatography-Mass Spectrometric (UPLC-MS)

The qualitative analysis of anthocyanin profiles was based on our previous work (Su et al., 2014). For anthocyanin extraction, 2 g of fresh hypocotyl samples were homogenized with 6 mL 1% HCl-methanol, and then the suspensions were placed in an ultrasonic bath for 10 min at 20°C. After centrifugation, 5 mL supernatant was taken out, and the residues were extracted with 6 mL extract solution again. The supernatants were combined. Then, daidzein was added as an internal standard to a final concentration of 0.03 μg mL⁻¹. After vacuum-drying, 500 μL of 80% methanol was added to dissolve the anthocyanin. And the supernatants were filtered through 0.22 μm membrane for further analysis.

The samples were analyzed by LC-MS system (G2-XS QToF, Waters). Two microliter solution was injected into the UPLC column (2.1 × 100 mm, ACQUITY UPLC BEH C18 column containing 1.7 μm particles) with a flow rate of 0.4 mL min⁻¹. The mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in acetonitrile. The solvent was administered according to the following protocol: 5% of B in 2 min, from 5 to 95% of B in 15 min, 95% of B in 2 min, and from 95 to 5% of B in 5 min. Mass spectrometry was performed using electrospray source in positive ion mode with MS acquisition mode, with a selected mass range of 50–1,200 *m/z*. The lock mass option was enabled using leucine-enkephalin (*m/z* 556.2771) for recalibration. The ionization parameters were the following: capillary voltage was 2.5 kV, collision energy was 40 eV, source temperature was 120°C, and desolvation gas temperature was 400°C. Data acquisition and processing were performed using Masslynx 4.1.

Determination of IP3 Content and CaM Content

IP3 extraction was measured according to the method of Burnette et al. (2003). Briefly, 0.5 g fresh sample of hypocotyl tissues were ground to a fine powder in liquid nitrogen. 0.5 mL 20% perchloric acid (v/v) was added and the mixture was incubated on ice for 20 min. After centrifugation (4°C, 2,000 g, 15 min), the precipitated protein was removed. The neutralized supernatant fraction was used to assay the IP3 content using an IP3 content assay kit (GE Healthcare) according to the manufacturer's instructions.

For CaM extraction, 0.5 g fresh hypocotyl samples were ground to a fine powder in liquid nitrogen. Then 2.5 mL extraction solution (50 mM tris-HCl containing 1 mM EGTA,

0.5 mM PMSF, and 1 mM β -mercaptoethanol) was added, and the mixture was incubated on ice for 20 min. After centrifugation at 12,000 g for 30 min at 4°C, the supernatant was taken out and incubated at 90°C for 3 min and then incubated on the ice immediately. The extraction was centrifuged at 12,000 g for 30 min at 4°C and the supernatant was collected for the measurement of protein and CaM content. For CaM content assay, plant CaM content assay kit (Kmaels) was used.

Determination of Enzyme Activities

For the analysis of enzyme activities, all operations were carried out at 4°C. The activity of anthocyanin-related enzymes in hypocotyl tissues referred to the enzyme activity in the crude protein fractions. PAL, CHS and CHI were extracted according to Li et al. (2013). For PAL and CHI extraction, 1 g of frozen radish hypocotyl tissues were ground to fine powder in liquid nitrogen and 3 mL of 100 mM Tris-HCl buffer (pH 8.8, containing 14 mM β -mercaptoethanol, 5 mM DTT, 1% BS and 5% PVPP) was added. The homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was used for PAL and CHI enzyme activity assay according to the methods of Ren and Sun (2014) and Lister et al. (1996), respectively. For CHS extraction, 2 g frozen radish hypocotyl tissues were homogenized with 5 mL of 100 mM sodium phosphate buffer (pH 6.8, containing 5% PVPP, 14 mM β -mercaptoethanol, 5 mM DTT, 40 mM sodium ascorbate, 10 μ M leupetin, 3 mM EDTA, and 2% BSA). The homogenate was centrifuged at 12,000 g for 20 min at 4°C. Then the supernatant was precipitated with ammonium sulfate [70% (w/v) saturation], kept on ice for 1 h, and centrifuged again. The resulting precipitate was dissolved in 1 mL of 100 mM sodium phosphate buffer (pH 6.8, containing 8 mM DTT, 40 mM sodium ascorbate, and 1% BSA). CHS activity was assayed using plant CHS activity assay kit (GE Healthcare) according to the manufacturer's instructions.

DFR crude protein were extracted according to the methods of Miyagawa et al. (2015). Briefly, 0.5 g frozen hypocotyl tissues were ground in liquid nitrogen and then suspended in 3 mL 0.1 M Tris-HCl buffer (pH 7.0), then centrifuged at 12,000 g for 15 min at 4°C. The proteins in the supernatant fraction were precipitated with 80% saturated ammonium sulfate and collected by centrifugation. The resulting precipitate was dissolved in 50 μ L of extraction buffer. The DFR activity was assayed using plant DFR ELISA kit (Janelly, Shanghai, China) according to the manufacturer's instructions. UFGT was extracted according to the methods of Li et al. (2013). UFGT activity were measured according to the methods of Zhang et al. (2014). Briefly, 10 μ L of enzyme extract was added to the reaction mixture, which contained 10 mM buffer (Hepes-KOH, pH 8.0), 250 mM MgCl₂, 2 mM DTT, 9 mM UDP-galactose, and 0.3 mM cyanidin, to a final volume of 200 μ L. The reaction mixture was incubated at 37°C for 10 min and terminated by the addition of 50 μ L 35% trichloroacetic acid. The UFGT activity was determined by HPLC at 525 nm.

Isolation of Total RNA and qRT-PCR

Total RNA was extracted from radish hypocotyls using Trizol reagent (Invitrogen, USA). One microgram of aliquots were

treated with RNase-free DNase I to remove genomic DNA and then reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's recommendation. qPCR was carried out on a Mastercycler ep realplex Real-time PCR System (Eppendorf, Hamburg, Germany) using Bestar SYBR Green qPCR Mastermix (DBI, Bioscience Inc., Germany) according to our previous methods (Wu et al., 2015). Reactions were performed at 95°C for 2 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The specific primers were designed according to the reference unigene sequence using the Primer Premier 6.0 software (see Table S1 in the Supplementary Material). *Actin2* and *EF1* were used as the reference genes (Xu et al., 2012). The relative expression levels of the selected genes were calculated using the $2^{-\Delta\Delta CT}$ approach, with normalization of data to the geometric average of two reference control genes (Vandesompele et al., 2002).

Statistical Analysis

Results were expressed as the means \pm SE (standard error) of at least three independent experiments. All the data were subjected to one-way ANOVA analysis (SPSS Statistics 17.0 software). Duncan's multiple range test was carried out to determine significant differences at $P < 0.05$.

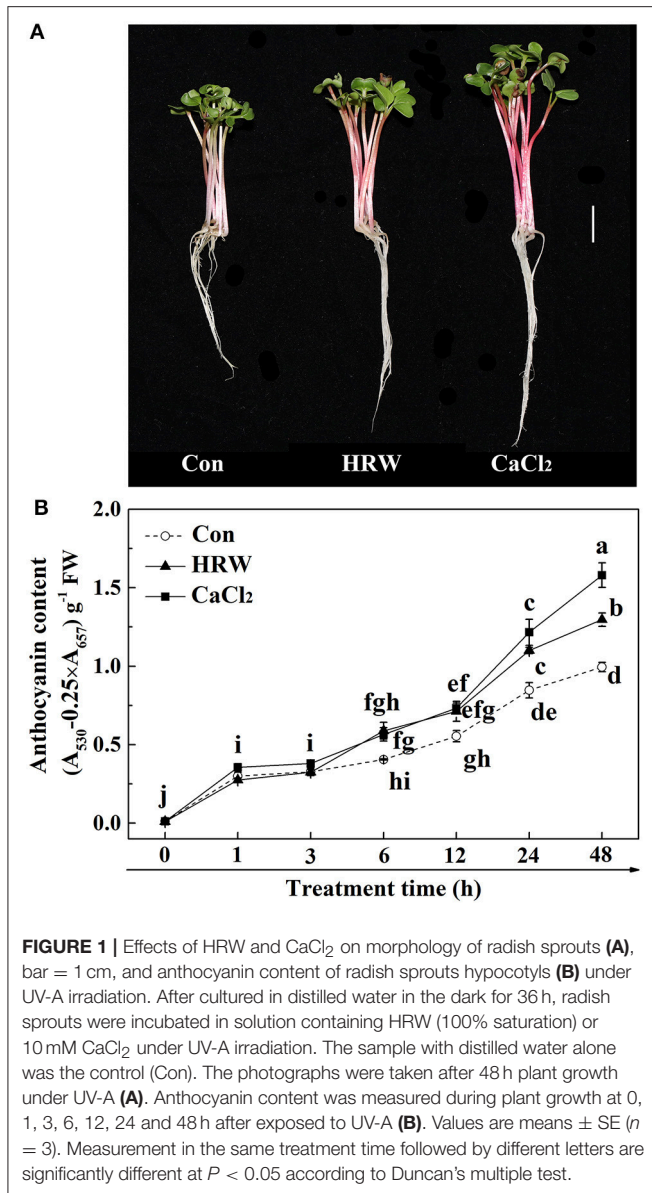
RESULTS

Anthocyanin Content Was Induced by HRW and CaCl₂ Under UV-A Irradiation

The effects of HRW and CaCl₂ on anthocyanin accumulation under UV-A irradiation were shown in **Figure 1**. In the control condition, the anthocyanin content in radish sprouts increased gradually during the culture time. In comparison to the control, both HRW and CaCl₂ treatments accelerated the increase of anthocyanin accumulation. Among them, the content of anthocyanin under CaCl₂ treatment at 12 h was slightly higher than that of the control. In addition, the differences in anthocyanin content between the two treatment groups and the control group increased as treatment time increased. At 48 h, the content of anthocyanin under HRW and CaCl₂ treatments were significantly higher than that of the control, and the content of anthocyanin under CaCl₂ treatment was the highest, significantly higher than that of other treatments.

HRW Promoted the Concentration of Cytosolic Calcium

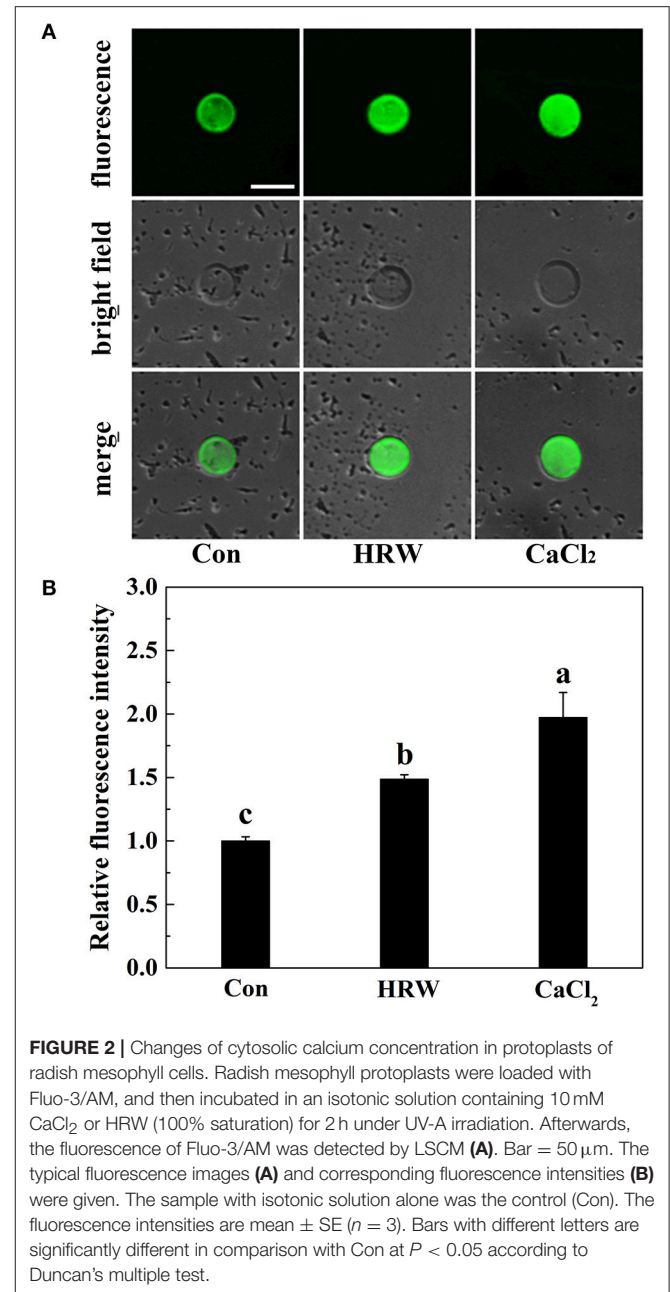
To investigate the possible role of cytosolic calcium in HRW-regulated anthocyanin accumulation in radish sprouts, we first examined the cytosolic calcium concentration. Radish mesophyll protoplasts treated with different reagents were loaded with calcium fluorescent probe Fluo-3/AM. The protoplasts of the control samples showed the basal cytosolic calcium concentration (**Figure 2**). Similar to the treatment of CaCl₂, HRW resulted in a significant increase in the cytosolic calcium concentration compared to control treatment. These results suggested that calcium may be one of the important signaling



components in HRW-triggered accumulation of anthocyanin under UV-A in radish sprouts.

HRW-Induced Anthocyanin Accumulation Was Blocked by the Specific Calcium Antagonists

Based on our hypothesis, calcium antagonists were exogenously applied. Compared with the control (UV-A treatment group), CaCl₂ addition significantly increased the anthocyanin content, while the calcium ion chelator EGTA, IP₃ biosynthesis inhibitor neomycin significantly reduced the anthocyanin content (Figure S2, Table 1). Whereas, calcium antagonists LaCl₃ and RR had no effects on anthocyanin content (Figure S2). These results indicated that IP₃-mediated calcium signal might take part in HRW-regulated anthocyanin biosynthesis. In the subsequent



experiment, EGTA and neomycin were chosen as calcium antagonists.

The Profile Analysis of Anthocyanin

Based on our previous experiments, the major anthocyanin components were determined by using UPLC-MS to evaluate the effects of CaCl₂, EGTA, and neomycin on HRW-promoted anthocyanin biosynthesis under UV-A. In this study, a total of 11 anthocyanins were detected in radish sprouts hypocotyls (Table 1), including cyanidin, delphinidin, pelargonidin and petunidin's derivatives. Among them, Cyanidin-3-O-rutinoside-5-O- β -D-glucoside and Cyanidin-3-O-rutinoside (two cyanidin derivatives) exhibited the highest abundance in radish sprouts

TABLE 1 | Anthocyanin profile of radish sprouts hypocotyls with different treatments under UV-A.

No.	Compound	Treatment							
		Con	Con + CaCl ₂	Con + EGTA	Con + Neomycin	HRW	HRW + CaCl ₂	HRW + EGTA	HRW + Neomycin
1	Cyanidin-3,5-O-diglucoside	0.3599 ± 0.0586c	0.1608 ± 0.0150d	0.1418 ± 0.0142d	0.1174 ± 0.0100d	0.3695 ± 0.0037c	1.0286 ± 0.0015a	0.6169 ± 0.0296b	0.3561 ± 0.0115c
2	Cyanidin-3-O-(6-O-(E)-4-coumaroyl-β-D-glucoside)	ND	ND	ND	ND	0.1909 ± 0.0031b	0.5550 ± 0.0248a	0.1633 ± 0.0048c	0.1378 ± 0.0031c
3	Cyanidin-3-O-(6-O-para-coumaroyl)glucoside-5-O-glucoside	ND	ND	ND	ND	0.1006 ± 0.0002b	0.1616 ± 0.0020a	ND	ND
4	Cyanidin-3-O-rutinoside 5-O-β-D-glucoside	2.8541 ± 0.0547d	10.0009 ± 0.0001b	2.3452 ± 0.1076ef	1.7116 ± 0.1245f	6.3203 ± 0.2363c	12.7610 ± 0.3597a	5.8785 ± 0.3455c	6.4453 ± 0.3606c
5	Cyanidin-3-O-rutinoside	5.9632 ± 0.1117e	21.3833 ± 0.0274b	3.3792 ± 0.1539f	4.1655 ± 0.3154f	15.5159 ± 0.2914c	23.2378 ± 0.1098a	14.2688 ± 0.3192d	6.4289 ± 0.7841e
6	Delphinidin-3-O-(6-O-(E)-4-coumaroyl-β-D-glucoside)	ND	0.2209 ± 0.0245a	ND	ND	0.1925 ± 0.0029a	0.2033 ± 0.0081a	0.1137 ± 0.0038b	ND
7	Delphinidin-3-O-arabinoside	0.2348 ± 0.0102c	0.3180 ± 0.0137b	0.2241 ± 0.0048c	0.1806 ± 0.0273d	0.3669 ± 0.0114a	0.3877 ± 0.0063a	0.2358 ± 0.0143c	0.1391 ± 0.0135d
8	Delphinidin-3-O-rutinoside	ND	0.1742 ± 0.0213d	ND	ND	0.1383 ± 0.0142e	1.0319 ± 0.0029a	0.6409 ± 0.0093b	0.3600 ± 0.0097c
9	Delphinidin-3-O-rhamnoside chloride	ND	ND	ND	ND	0.1340 ± 0.0012a	0.1365 ± 0.0021a	ND	ND
10	Pelargonidin-3-O-glucoside	0.2690 ± 0.0183d	1.2818 ± 0.1164b	0.1511 ± 0.0038d	0.1563 ± 0.0270d	1.4600 ± 0.0234b	2.4378 ± 0.154a	1.0307 ± 0.0086c	2.2313 ± 0.1189a
11	Petunidin-3-O-galactoside	0.0393 ± 0.0107d	0.2273 ± 0.0074a	0.2303 ± 0.0106a	0.1299 ± 0.0146b	0.1060 ± 0.0073bc	0.1236 ± 0.0072bc	0.1000 ± 0.0000c	ND
	Total anthocyanin	9.7202 ± 0.2198f	33.7672 ± 0.1034b	6.4384 ± 0.1613g	6.4614 ± 0.4336g	24.8948 ± 0.5535c	42.0647 ± 0.3134a	23.0488 ± 0.7105d	16.0984 ± 0.8664e

Data are presented as means ± SE (n = 3).

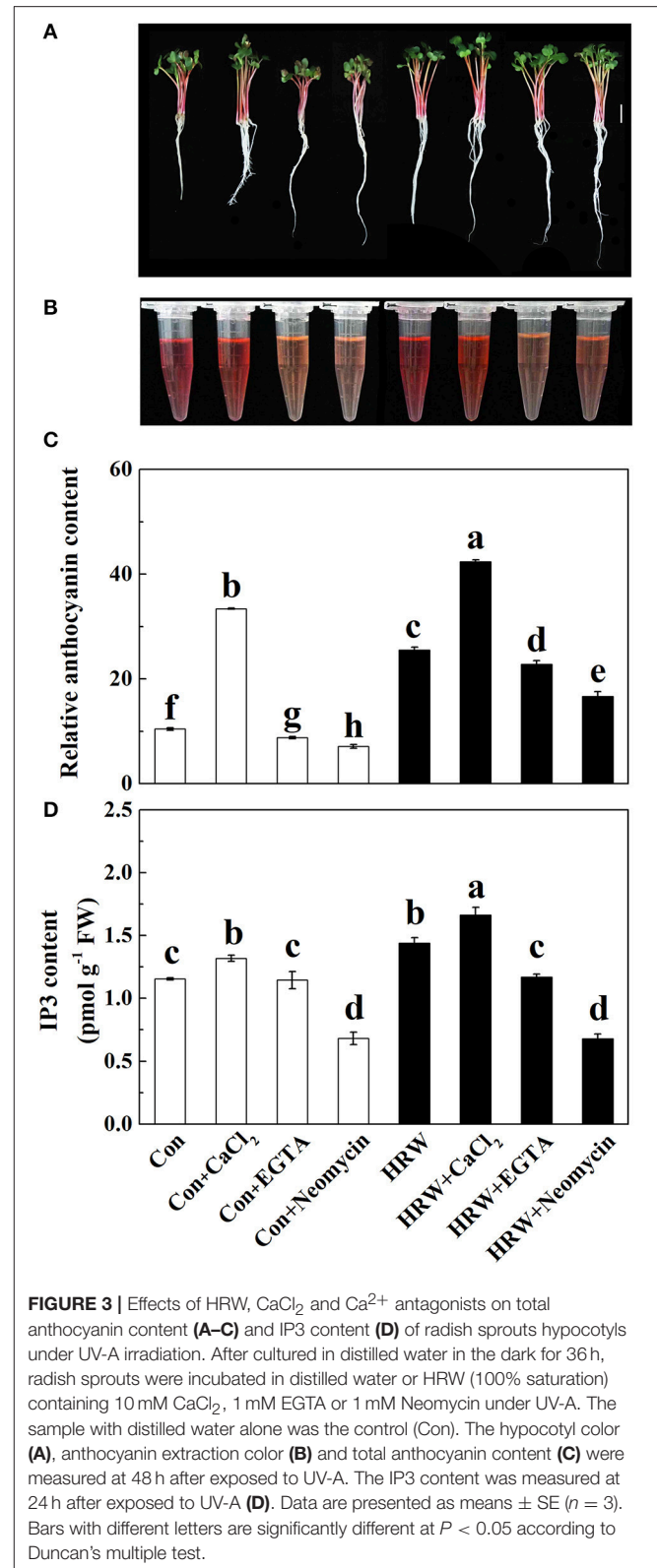
Values followed by different letters are significantly different according to Duncan's multiple test at P < 0.05. ND, Not Detectable.

hypocotyls, followed by Cyanidin-3,5-*O*-diglucoside and Pelargonidin-3-*O*-glucoside. In the absence of HRW, CaCl₂ increased the anthocyanin components from 6 to 8. Compared with the control, CaCl₂ significantly increased the content of most of the anthocyanin components. For example, the content of Cyanidin-3-*O*-rutinoside-5-*O*-β-D-glucoside and Cyanidin-3-*O*-rutinoside under CaCl₂ treatment was 3.50- and 3.59-fold higher than the control, respectively. Whereas EGTA and neomycin significantly decreased both the components and content of anthocyanins. In comparison to the control, HRW increased the components of anthocyanins to 11 and significantly increased the content of Cyanidin-3,5-*O*-diglucoside, Cyanidin-3-*O*-rutinoside and Pelargonidin-3-*O*-glucoside. It is also important to point out that the amount of the above three anthocyanins were further increased by HRW + CaCl₂ treatment. However, the co-treatment of HRW with EGTA or neomycin decreased the number of anthocyanins and significantly inhibited anthocyanin contents. Besides, some anthocyanin monomer contents were significantly decreased by EGTA and neomycin, whether HRW was presence or not. For instance, in the absence of HRW, the content of cyanidin-3-*O*-rutinoside upon EGTA and neomycin treatment accounted for 62 and 57% of that of the control, respectively. While in the presence of HRW, the content of cyanidin-3-*O*-rutinoside upon EGTA and neomycin treatment accounted for 92 and 41% of that of HRW treatment, respectively.

Further analysis showed that the total anthocyanin content was significantly increased upon CaCl₂ treatment (3.47-fold than the control), but significantly decreased upon EGTA and neomycin treatment (decreased by 33.76 and 33.52% than the control, respectively). It was noteworthy that the total anthocyanin content was significantly increased when treated with HRW (2.56-fold than the control) and was further significantly increased when treated with HRW + CaCl₂ (4.33-fold than the control). However, when treated with HRW + EGTA and HRW + Neomycin, total anthocyanin content significantly decreased than the HRW treatment. Thus, these results revealed that both the component and content of anthocyanin in radish sprouts were affected by CaCl₂ and calcium antagonists (EGTA and neomycin). It further suggested that IP₃-mediated calcium signal plays an important role in HRW-regulated anthocyanin biosynthesis under UV-A.

Effects of CaCl₂ and Calcium Antagonists on IP₃ Content

To confirm the role of IP₃ in HRW-promoted anthocyanin biosynthesis, IP₃ content was measured. Compared with the control, IP₃ content was significantly increased with CaCl₂, but significantly decreased with neomycin treatment (Figures 3A–C). In the presence of HRW, the same phenomenon was observed. We also noticed that the incubation of radish sprouts with EGTA, whether HRW was present or not, had no effects on IP₃ content (Figure 3C). However, EGTA decreased total anthocyanin content in HRW-treated samples (Figures 3B,C). Overall, the trend of IP₃ content in all treatments coincided with that of total anthocyanin content (Figure 3).



These results indicated that IP₃-dependent calcium signaling pathway might be involved in HRW-regulated anthocyanin biosynthesis under UV-A.

Activation of Anthocyanin Biosynthetic Enzymes

The analysis of anthocyanin biosynthetic enzymes showed that CaCl_2 significantly increased the activity of PAL, CHS, CHI, and DFR in the crude protein extractions prepared from the hypocotyl tissues, whereas EGTA and neomycin significantly decreased the activity of PAL, CHI, and DFR, compared with the

control (Figures 4A–D). HRW significantly increased the activity of PAL and DFR, which was further increased by HRW + CaCl_2 (Figures 4A,D). Meanwhile, HRW-induced increase of PAL, CHI and DFR activity was decreased by EGTA and neomycin (Figures 4A,C,D). Besides, HRW-induced CHS activity was significantly decreased by neomycin (Figure 4B). For the activity of UFGT, CaCl_2 , and HRW significantly increased UFGT activity.

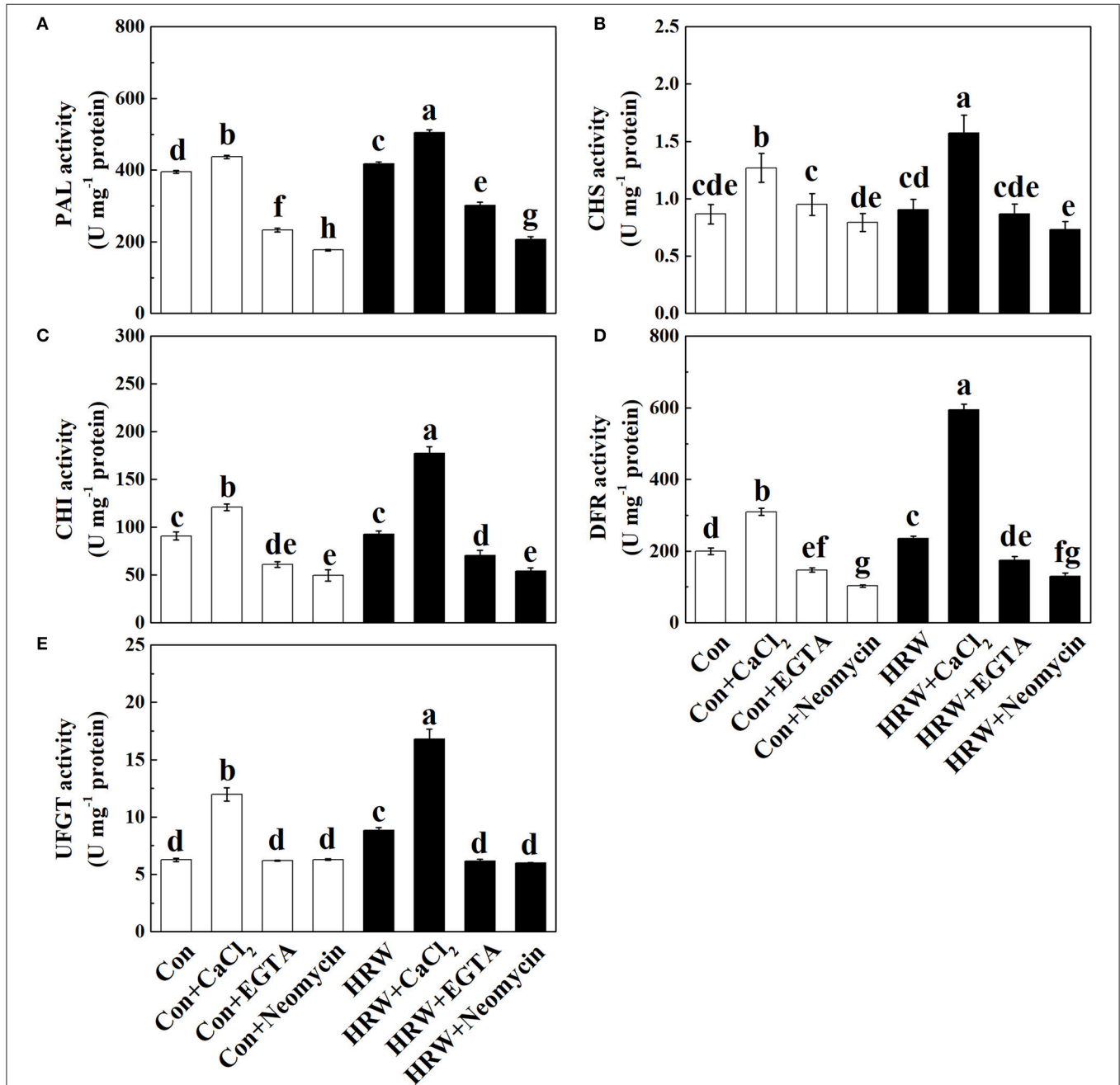


FIGURE 4 | Effects of HRW, CaCl_2 and Ca^{2+} antagonists on activity of anthocyanin biosynthesis-related enzymes in radish sprouts hypocotyls under UV-A irradiation. Radish sprouts were further incubated in distilled water or HRW (100% saturation) containing 10 mM CaCl_2 , 1 mM EGTA or 1 mM Neomycin under UV-A irradiation. The sample with distilled water alone was the control (Con). The activity of PAL (A), CHS (B), CHI (C), DFR (D) and UFGT (E) were measured at 24 h plant growth under UV-A. Data are presented as means \pm SE ($n = 3$). Bars with different letters are significantly different at $P < 0.05$ according to Duncan's multiple test.

Meanwhile, compared to HRW treatment, HRW + CaCl₂ further increased UFGT activity. However, EGTA and neomycin significantly decreased UFGT activity only in the presence of HRW.

Transcript Levels of Anthocyanin Biosynthetic-Related Genes

To further provide the molecular basis for above anthocyanin profile changes, the transcript levels of genes responsible for anthocyanin biosynthesis were analyzed by qRT-PCR. As shown in **Figure 5**, CaCl₂ caused an increase in the expression level of all the genes detected (except for *RsLDOX*). These increasing tendencies were reversed by EGTA and neomycin. It is also important to note that the expression of two MYB transcription factors *RsPAP1* and *RsPAP2* was significantly promoted by HRW, and further promoted by HRW + CaCl₂ (**Figures 5I,J**). Compared with the control, HRW significantly increased the expression of all the key genes. Furthermore, these promoting effects were substantially strengthened by CaCl₂ upon HRW treatment (except for *RsUFGT*). However, the expression levels of these key genes were down-regulated when EGTA or neomycin was co-treated with HRW. In general, the transcript levels of these key genes were in accordance with the changes of total anthocyanin content in radish sprouts hypocotyls.

The Possible Involvement of CaM in HRW-Regulated Anthocyanin Biosynthesis

To determine the role of CaM in HRW-regulated anthocyanin synthesis under UV-A, the dynamic changes of CaM abundance were measured (**Figure 6**). The results showed that the content of CaM significantly increased at 6 and 12 h incubation in HRW compared with the control, but significantly decreased when neomycin was co-treated. Similarly, CaM content significantly increased after 6 and 12 h incubation in CaCl₂, and significantly decreased when neomycin was co-treated. Meantime, CaM content significantly decreased after incubation with Neomycin at 3, 6, and 12 h. To further clarify the involvement of CaM in HRW-regulated anthocyanin accumulation under UV-A, CaM inhibitor W7, W5, and TFP were exogenously added. As shown in **Figure S3**, CaM inhibitors W7 and TFP significantly reduced anthocyanin content. However, W5, a structural analog of W7, had no any effect on anthocyanin content.

DISCUSSION

Hydrogen gas was once considered as an inert gas that has no physiological effect on human body or plants. However, increasing number of studies in both mammalian and plant system have changed our previous standard stereotypes (Ohsawa et al., 2007; Ohta, 2012; Jin et al., 2013; Su et al., 2014; Chen et al., 2017). Recently, Xie et al. (2015) found that HRW could partially alleviate UV-B-induced oxidative damage by the manipulation of (iso)flavonoids metabolism in alfalfa. Our previous study also showed that HRW could increase UV-A-induced anthocyanin biosynthesis in radish sprouts hypocotyls

(Su et al., 2014). However, the underlying mechanisms of HRW-regulated anthocyanin biosynthesis need to be fully clarified.

The biosynthesis of anthocyanin is regulated by environmental and developmental signals (Jaakola, 2013). Ca²⁺, a universal second messenger, plays an important role in the transduction of growth and development signaling, environmental signaling and hormone signaling (Yang and Poovaliah, 2003; Hetherington and Brownlee, 2004; Batistić and Kudla, 2012). The importance of Ca²⁺ for improving or maintaining fruit quality has long been recognized (Oms-Oliu et al., 2010; Zhi et al., 2017). However, there are relatively few studies on the effects of Ca²⁺ on the metabolism of anthocyanins and flavonoids. In this study, we provided strong evidence to illustrate the role of Ca²⁺ in HRW-induced anthocyanin biosynthesis in radish sprouts exposed to UV-A irradiation.

Firstly, we observed that HRW significantly increased the anthocyanin content, which mimicked the inducing effect of CaCl₂ on the anthocyanin content (**Figure 1**). This result was consistent with a previous study in strawberry (Xu et al., 2014), showing that Ca²⁺ enhanced anthocyanin accumulation. Meanwhile, similar to the effect of CaCl₂, the cytosolic Ca²⁺ concentration was significantly increased by HRW (**Figure 2**). Our results suggested that Ca²⁺ plays an important role in HRW-induced anthocyanin biosynthesis.

Secondly, the pharmacological experiments showed that Ca²⁺ chelator EGTA and IP₃ synthesis inhibitor neomycin significantly inhibited the HRW-induced anthocyanin accumulation under UV-A (**Figure S2, Table 1**). These results were consistent with recent reports (Shin et al., 2013; Jiao et al., 2016), showing that EGTA and neomycin decreased anthocyanin content in *Arabidopsis* and soybean sprouts. It has been reported that soybean sprouts produced by soaking Ca²⁺ and spraying Ca²⁺ contained more isoflavone (Wang et al., 2016). The present study showed that Ca²⁺ and calcium antagonists affect both the composition and content of anthocyanins (**Table 1**). To an extent, the content of various anthocyanin monomers showed a similar trend under different treatments, that is the anthocyanin monomers content increased under CaCl₂ treatment and decreased under EGTA and neomycin treatments, whether in the presence of HRW or not. Similar inhibition of isoflavone production was previously reported in soybean sprouts, showing that UV-B-induced isoflavone production in soybean sprouts was hindered by neomycin (Jiao et al., 2016). It was also noted that the total anthocyanin content increased significantly under CaCl₂ treatment but decreased significantly under EGTA and neomycin treatments in the presence of HRW (**Table 1**). Meanwhile, the IP₃ content under HRW treatment was significantly higher than that of the control and was significantly decreased under neomycin treatment (**Figure 3**), implying that HRW induced IP₃ accumulation in radish sprouts hypocotyls. The above results indicated that Ca²⁺ signaling participates in the regulation of anthocyanin biosynthesis triggered by HRW, of which IP₃-sensitive Ca²⁺ channels play a major role in our experimental conditions.

Thirdly, previous studies revealed that PAL, CHS, and CHI are the important early key genes in anthocyanin biosynthesis (Holton and Cornish, 1995; Jez et al., 2000; Rohde et al., 2004;

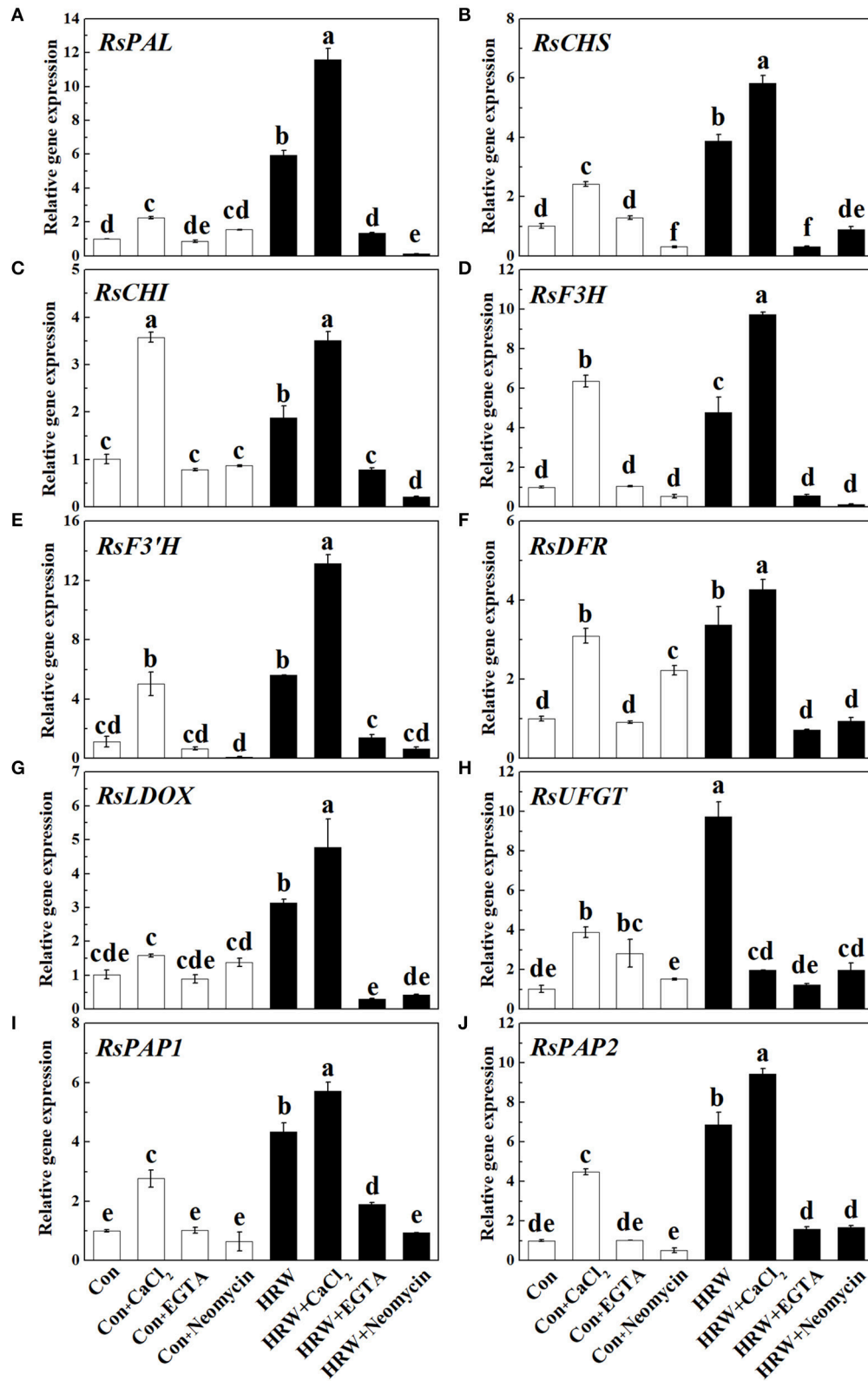
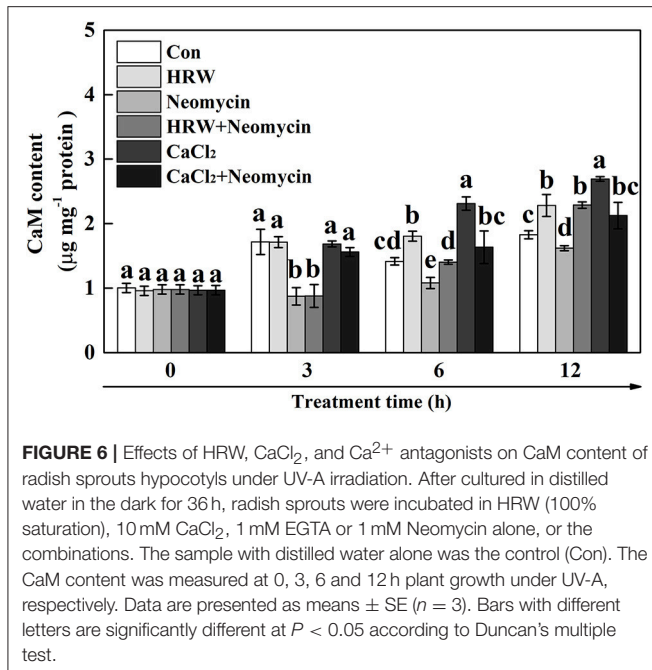
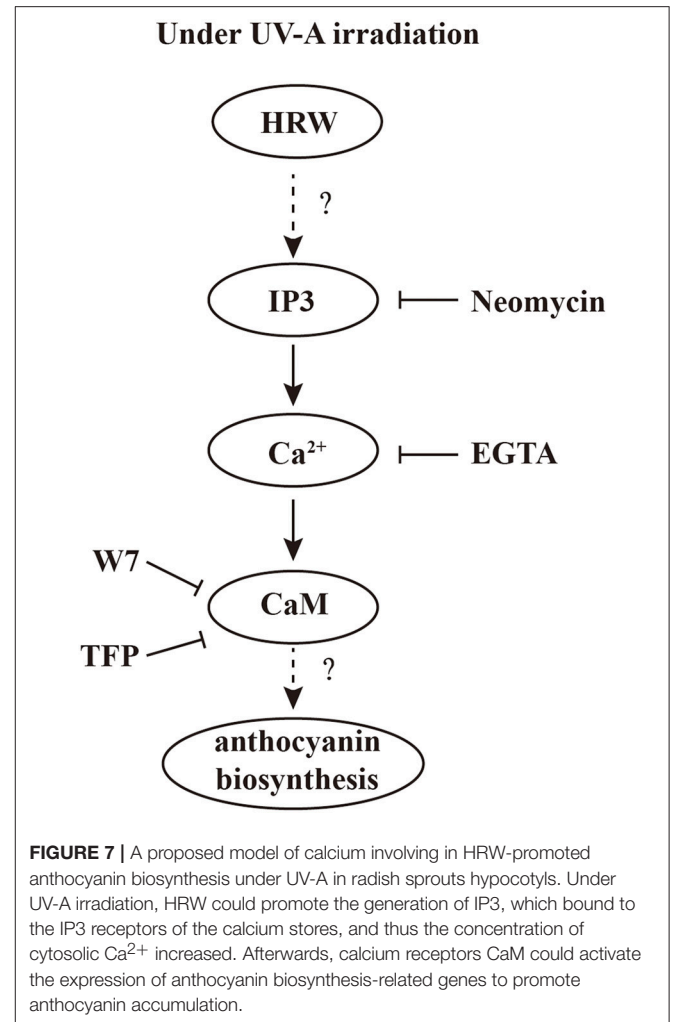


FIGURE 5 | Expression of genes related to anthocyanin biosynthesis in radish sprouts hypocotyls under UV-A irradiation. Radish sprouts were further incubated in distilled water or HRW (100% saturation) containing 10 mM CaCl₂, 1 mM EGTA or 1 mM Neomycin. The sample with distilled water alone was the control (Con). The gene expression levels of *RsPAL* (A), *RsCHS* (B), *RsCHI* (C), *RsF3H* (D), *RsF3'H* (E), *RsDFR* (F), *RsLDOX* (G), *RsUFGT* (H), *RsPAP1* (I) and *RsPAP2* (J) were analyzed by qRT-PCR at 12 h plant growth under UV-A. Data are presented as means ± SE (*n* = 3). Bars with different letters are significantly different at *P* < 0.05 according to Duncan's multiple test.



Li et al., 2010), and DFR and UFGT are two important enzymes that catalyze the late steps of anthocyanin biosynthesis (Gonzalez et al., 2008). In the present study, the activity tendencies of the above mentioned key enzymes showed similar tendency of anthocyanin content (Figures 3, 4). Also, it has been suggested that *CHS*, *CHI*, *F3H*, and *F3'H* are the early biosynthetic genes (EBGs) in the flavonoid biosynthesis pathway which involved in the production of common precursors. And the downstream late biosynthetic genes (LBGs), including *DFR*, *LDOX*, and *UFGT*, are positively regulated by MYB transcription factors (Jaakola, 2013; Xu et al., 2015). In this study, the molecular evidence showed that the upregulation of *RsDFR*, *RsLDOX*, *RsUFGT*, *RsPAP1*, and *RsPAP2* induced by HRW were promoted after treated with CaCl₂ but reversed after EGTA and neomycin treatments (Figures 5F–J). It is noteworthy that the expression level of upregulated genes (i.e., *RsPAL*, *RsCHS*, *RsCHI*, *RsF3H*, and *RsF3'H*) showed a similar expression pattern to the content of anthocyanin (Figures 5, 3C). This result is contrary to previous studies showing that MBW complexes regulate LBGs but not the EBGs nor the *PAL* (Koes et al., 2005; Jaakola, 2013). It has been well documented that PAP family is required specifically for anthocyanin accumulation and provides specificity to the MBW complex (Borevitz et al., 2000; Maier et al., 2013). Overexpressing *PAP1* and *PAP2* could promote anthocyanin accumulation in *Arabidopsis* and tobacco by up-regulating the entire phenylpropanoid pathway (Gonzalez et al., 2008; Mitsunami et al., 2014). In this study, the higher expression of *PAP1* and *PAP2* in radish sprouts treated with exogenous application of Ca²⁺ could, at least partly, explain the positive effect of exogenous Ca²⁺ on anthocyanin accumulation.

CaM was involved in sugar-induced anthocyanin biosynthesis in *Vitis vinifera* cells (Vitrac et al., 2000). Furthermore, changes of CaM activity were directly correlated with



anthocyanin accumulation under low temperature in *A. bettzickiana* (Wang et al., 2005). Another research showed that calcium/calmodulin specifically bound FvUGT1 to enhance anthocyanin accumulation in strawberry (Peng et al., 2016). In this study, the promotion effect of HRW on CaM content is partially inhibited by neomycin (Figure 6). Subsequent pharmacological experiments showed that CaM inhibitor W7 and TFP significantly decreased anthocyanin content (Figure S3). These results provided the evidence for the contribution of CaM in terms of promoting HRW-triggered anthocyanin biosynthesis. Our results indicated that CaM probably takes part in calcium signal transduction to regulate HRW-triggered anthocyanin biosynthesis under UV-A.

CONCLUSION

To our knowledge, using pharmacological and molecular approaches, which, in the case of multi-parameters, our data provide the first evidence that calcium signal plays an important role in HRW-triggered anthocyanin biosynthesis under UV-A (Figure 7). Under UV-A irradiation, HRW could promote the generation of IP3, which bind to the IP3 receptors of the

calcium stores, and thus the concentration of cytosolic Ca^{2+} increased. Afterwards, calcium receptors CaM could activate the expression of anthocyanin biosynthesis-related genes to promote anthocyanin accumulation. In our study, the MYB transcription factors RsPAP1 and RsPAP2 played dominantly roles in this process. However, the interaction of calcium signaling and MBW complex should be further investigated. Moreover, the corresponding molecular evidence should be provided to uncover the targets of calcium signal, and then to well illustrate the regulation pattern of HRW-triggered anthocyanin biosynthesis under UV-A.

AUTHOR CONTRIBUTIONS

JC and XZ conceived and designed the study. XZ and JW performed the experiments and analyzed the data. YH helped in performing the experiments. XC and WS provided the experimental methods. XZ wrote the manuscript, which was revised by NS and CL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01020/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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