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Research article

Regulation of flavanone 3-hydroxylase gene involved in the flavonoid biosynthesis pathway in response to UV-B radiation and drought stress in the desert plant, *Reaumuria soongorica*

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ABSTRACT

Flavonoid are known to have various functions in growth, development, reproduction, and also involved in diverse stress responses in plants. However, little is known about the roles of the key enzymes in the flavonoid biosynthetic pathway in response to environmental stress, such as UV-B radiation and drought. To understand this problem, we investigated the participation of flavanone 3-hydroxylase gene (F3H), a key enzyme in flavonoid biosynthetic pathway under UV-B radiation and drought stress in the desert plant *Reaumuria soongorica*. A novel cDNA sequence, named as *RsF3H*, was isolated from *R. soongorica*. The deduced amino acids showed high identities to other F3Hs. A phylogenetic analysis indicated that *RsF3H* appeared to be most homologous to F3H from *Malus domestica* (*MdF3H*). *RsF3H* protein structure contained all five conserved motifs for 2-oxoglutarate-dependent dioxygenases (2-ODDs) and an Arg-X-Ser motif, all of which were also found in other F3Hs. Quantitative real-time RT-PCR analysis showed that there was a rapid increase in gene expression of *RsF3H* under stress. Both UV-B radiation and drought stress induced an increase in *RsF3H* enzyme activity and the accumulation of the products in the flavonoid biosynthetic pathway (total flavonoid and anthocyanin). The antioxidant ability (inhibition of lipid oxidation) of total flavonoid was enhanced during this study. The results suggested that one explanation of the stress tolerance of *R. soongorica* may be a combination of an increase in *RsF3H* gene expression, *RsF3H* enzyme activity and the anti-oxidative ability of the metabolic end products in the flavonoid biosynthetic pathway in response to UV-B radiation and drought.

1. Introduction

Flavonoid, a class of low-weight phenolic compounds, are widely distributed in plants. Attributed to their structural diversity, flavonoid perform varied biological functions, such as acting as pigments to attract pollinators, as auxin transport regulators and as molecular signals for the interaction of plants with microorganisms [1]. Most importantly, flavonoid are involved in the responses to biotic and abiotic stress [2]. Over the years, flavonoid always have attracted the attention of many scientists. The flavonoid biosynthetic pathway has been thoroughly investigated [1]. A collection of mutant lines defective in flavonoid biosynthetic pathway have been identified in *Arabidopsis* on the basis of altered color of seed coat [3]. The majority of enzymes in the pathway have been identified from a number of different species [4–6]. Flavonoid biosynthetic genes, such as chalcone synthase (CHS), chalcone isomerase (CHI), flavone synthase II (FSII), flavonoid 3-hydroxylase (F3′H) and flavanone 3-hydroxylase (F3H), have been used to modify flower color [7–10]. F3H is one of the first three genes (CHS, CHI, F3H) encoding the early, unbranched segment of the flavonoid biosynthetic pathway. F3H is a key enzyme in the flavonoid biosynthetic pathway, catalyzing the 3-hydroxylation of (2*S*)-flavanones, such as naringenin to dihydroflavonols. The F3H gene has been cloned from many other species [5,8,11,12].

Abbreviations: F3H, flavanone 3-hydroxylase; 2-ODDs, 2-oxoglutarate-dependent dioxygenases; RACE, rapid amplification of cDNA ends; TDR, time domain reflectometry; ORF, open reading frame; TBA, thiobarbituric acid; MDA, malonaldehyde; qRT-PCR, quantitative real-time RT-PCR; ROS, reactive oxygen species; HPLC, high performance liquid chromatography.

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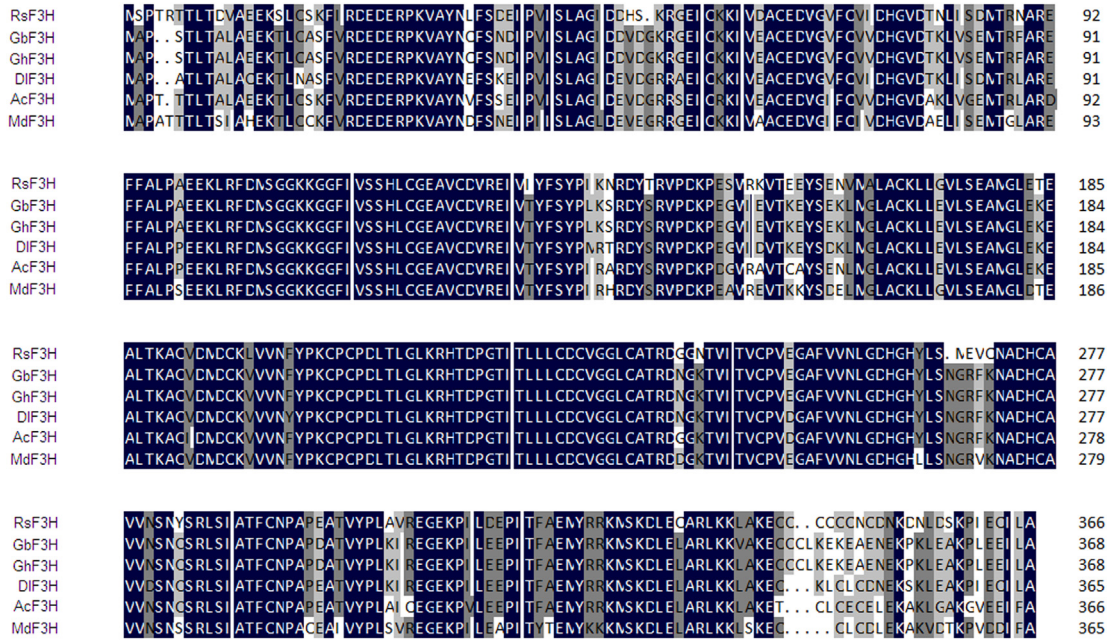

Fig. 1. Alignment of *RsF3H* with F3H proteins from other species. Genbank accession numbers for the proteins in the alignment are as follows: *GbF3H* (ABL86673), *GhF3H* (ABM64799), *DlF3H* (AB048521), *AcF3H* (ACL54955) and *MdF3H* (AAX89398). The sequences were aligned using DNAMAN software.

Some recent studies have focused on the stress protection role of flavonoid, especially their involvement in responses to abiotic signals, including UV damage, temperature fluctuations and low availability of nutrients and water [13]. Flavonoid were found to accumulate rapidly and had a protective role when plants were exposed to UV-B radiation or drought [14,15]. It is reported that flavonoid are important UV-B shielding compounds due to their absorbance in this wavelength region and the significant increase in their concentrations in epidermal layers under UV radiation [16]. Flavonoid may also inhibit oxidative stress induced by environmental stress [17]. Up-regulation of the flavonoid pathway-related genes has been observed in potato, birch and rice after stress exposure [18–20]. These studies indicated that the flavonoid pathway may be involved in the response to stress. However, the roles of the key enzymes and the regulatory mechanism involved in the response to abiotic stresses in the flavonoid biosynthetic pathway are still poorly understood.

Reaumuria soongorica (Pall.) Maxim., a super-xerophytic desert semi-shrub, is a typical constructive and dominant species of desert vegetation community and is widely distributed in northwestern China [21]. As a resurrection plant, *R. soongorica* withers and enters a state of dormancy when desiccation. When precipitation occurs, it revives and will continue its life cycle [22]. It can survive in severe environments, showing tolerance to drought, salinity, extreme temperature fluctuations and UV irradiation. In recent years, several studies have focused on genetic diversity, the protection mechanism for photosynthetic components and metabolite changes [21]. Until now, few studies have been carried out on the effect of abiotic stress on the molecular regulation of primary and secondary metabolism in *R. soongorica*.

In the present paper, a gene (*RsF3H*) of the flavonoid biosynthetic pathway was isolated from *R. soongorica* using the rapid amplification of cDNA ends (RACE) method. The expression levels of the *RsF3H* gene, enzyme activity, the contents of total flavonoid and anthocyanin and the antioxidant ability of flavonoid were investigated under UV-B radiation and drought stress. The results of this study are expected to enable us to explore the stress-tolerance mechanism behind flavonoid participating in environmental stress responses in *R. soongorica*.

2. Results

2.1. Characterization of RsF3H

The full-length cDNA of *RsF3H* was 1409 bp and contained an 1101 bp ORF. The entire sequence has been deposited in the Genbank database with accession number JQ043380. It encoded a protein consisting of 366 amino acids with a theoretical molecular mass of 41.35 kDa and an isoelectric point of 5.01. The hydrophobicity profile of the *RsF3H* protein was predicted and the plot analysis showed that *RsF3H* was highly hydrophilic and had no predicted trans-membrane domain. Alignment of predicted amino acid sequences showed that the *RsF3H* protein contained five similar motifs for 2-ODDs [12], which were also found in other F3H proteins (Fig. 1). Among the five motifs, motifs 1, 2, 3 and 4 of *RsF3H* were absolutely conserved compared to the other plants (Fig. 1), but several amino acids had diverged in motif 5. Three prolines were conserved strictly in motifs 2 and 3, which were predicted to play crucial roles in the process of polypeptide folding. There were four amino acid residues (His76, His218, Asp220 and His275) for binding ferrous iron and an Arg285-X-Ser287 motif (RXS) that takes part in 2-oxoglutarate binding in *RsF3H* [12]. All of these were found in similar positions compared to their locations in F3Hs found in other plants (Fig. 1). All the conservation observed in these amino acids suggested that *RsF3H* protein have potential biological function. A phylogenetic analysis indicated that *RsF3H* appeared to be most homologous to F3H from *Malus domestica* (*MdF3H*) (Fig. 2).

2.2. Soil and leaf water content

The soil water content dropped dramatically from 34.6% to 4.94% during drought treatment. And the leaf water content decreased with the dropping of the soil water content (Fig. 3A). Leaf

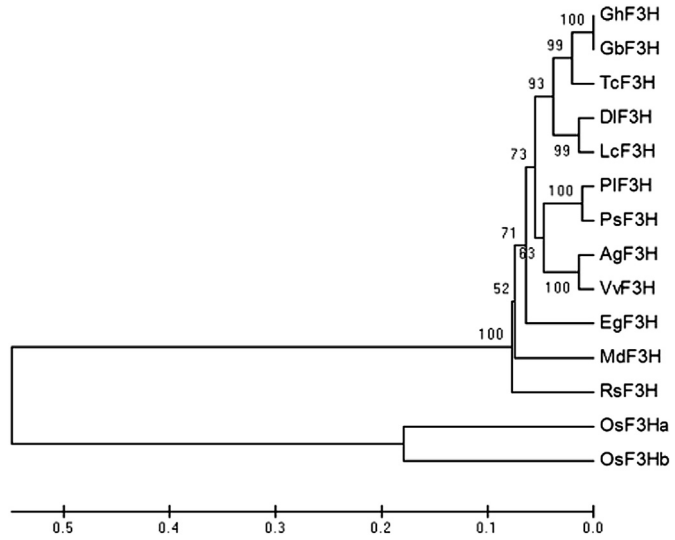

Fig. 2. The phylogenetic tree. Genbank accession numbers of F3H proteins used for construction of the tree are as follows: *GhF3H* (AMB64799), *GbF3H* (ABL86673), *TcF3H* (EOX90854), *DlF3H* (ABO48521), *LcF3H* (ADO95201), *PlF3H* (AFI71897), *PsF3H* (AEN71544), *AgF3H* (AFN70721), *VvF3H* (NP_001268034), *EgF3H* (BAD34459), *MdF3H* (AAX89398), *RsF3H* (AEY81365), *OsF3Ha* (NP_001048799), *OsF3Hb* (NP_001053651).

water content was decreased by approximately 20% after 10 days of drought treatment.

2.3. Effect on lipid peroxidation and chlorophyll content

The lipid peroxidation is often taken as an index of oxidative damage and estimated as malondialdehyde (MDA) accumulation [23]. Both treatments significantly increased the MDA content in *R. soongorica* during 10 days (Fig. 3B). After 10 days of treatment, exposure to UV-B increased the accumulation of MDA by approximately 52%. Under drought stress, the content of MDA was increased by 107% over untreated control plants. The chlorophyll content (Fig. 3C) decreased significantly under UV-B radiation and drought during the experiment.

2.4. RsF3H gene expression and enzyme activity analysis

The expression levels were analyzed by qRT-PCR. The expression of *RsF3H* increased rapidly after exposure to UV-B for 4 days, and the maximum expression level reached was up to 3.5 times greater than that in the control group. Then its expression level decreased after 4 days treatment, but it remained higher than that observed in the control group (Fig. 4A). During exposure to drought, the variation of the expression level of *RsF3H* was similar to that under UV-B radiation. *RsF3H* activity increased sharply within 6 and 4 days after exposure to UV-B radiation treatment and drought treatment, respectively, and then, in both treatment groups, showed decreasing trends (Fig. 4B).

2.5. Variations in total flavonoid, anthocyanin contents and inhibition of lipid oxidation

The total flavonoid content declined within 4 days and then increased under UV-B radiation treatment (Fig. 4C). Under UV-B radiation treatment, the anthocyanin content increased consistently throughout the study period (Fig. 4D). The inhibition of lipid oxidation of flavonoid showed the same trend as the anthocyanin content (Fig. 4E). Under drought treatment, the total flavonoid and anthocyanin content rose throughout the study period (Fig. 4C, D). Moreover, the inhibition of lipid oxidation of flavonoid increased consistently under the drought treatment (Fig. 4E).

3. Discussion

We report identification and isolation of the first full-length F3H cDNA (*RsF3H*) from the desert semi-shrub *R. soongorica*. All F3Hs

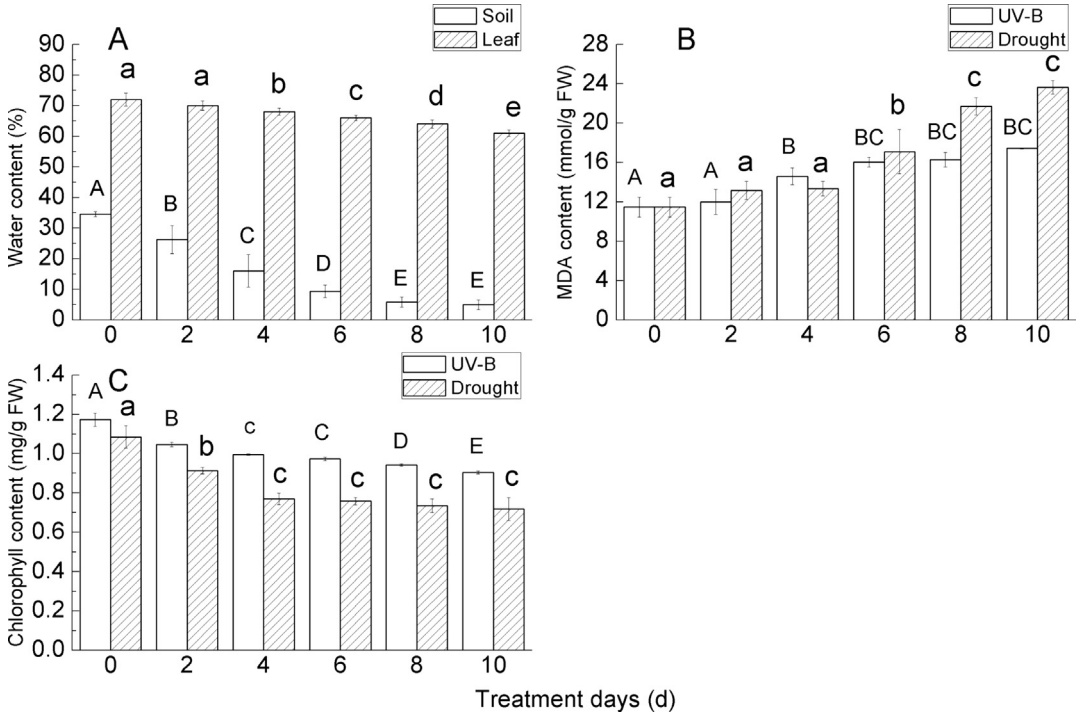

Fig. 3. Variations of (A) soil and leaf water content under drought, (B) MDA levels and (C) chlorophyll contents under UV-B radiation and drought stress. The controls were noted as 0 day of treatment.

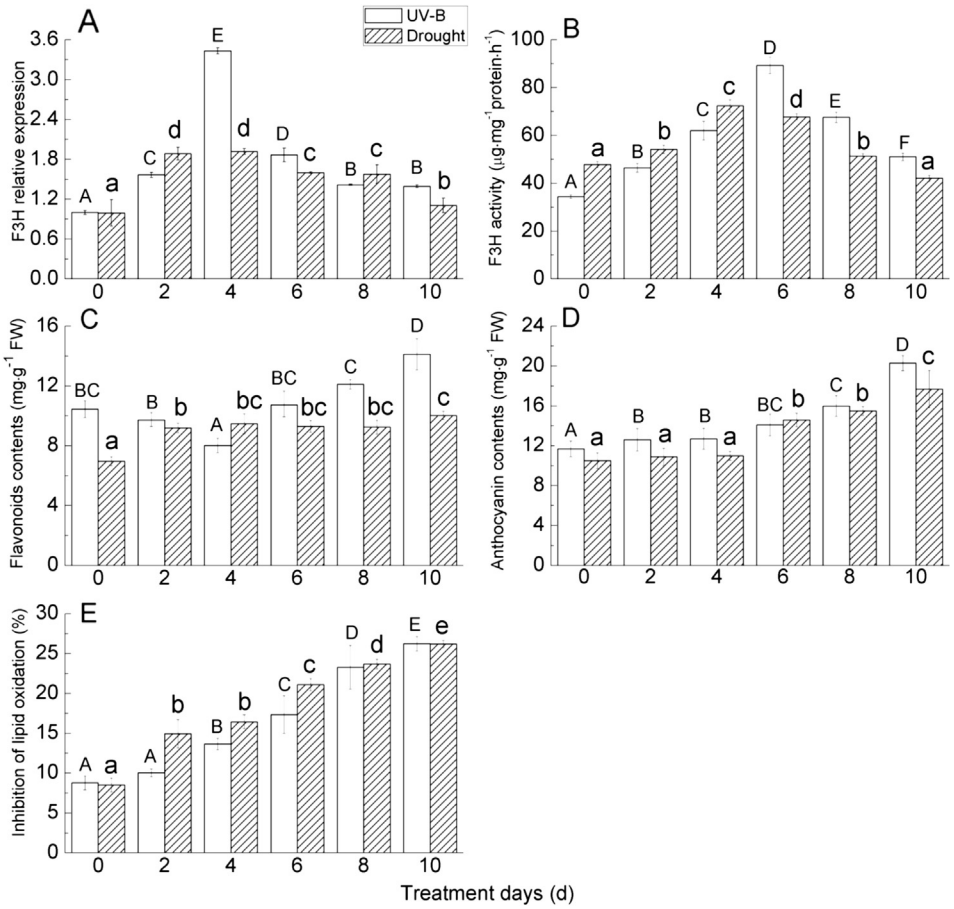

Fig. 4. Variations in (A) *RsF3H* expression level, (B) *RsF3H* activity, (C) total flavonoid content, (D) anthocyanin content and (E) inhibition of lipid oxidation under UV-B radiation and drought stress. The controls were represented as 0 day of treatment.

contain seven conserved residues for 2-ODDs, which consist of two motifs: His-His-Asp-His for ligating Fe^{2+} and RXS for binding oxoglutarate [12]. These conserved residues were also observed in *RsF3H* (Fig. 1). A phylogenetic analysis implies the evolution relationship of *RsF3H* and other orthologs in plants and the result showed that *RsF3H* appeared to be most homologous to *MdF3H* (Fig. 2).

Abiotic stress leads to the disturbance of plant metabolism and also causes oxidative damage by enhancing the generation of reactive oxygen species (ROS). One result of ROS accumulation in plant induced by stress is lipid peroxidation via oxidation of unsaturated fatty acids, resulting in electrolyte leakage and membrane damage [24]. MDA is the product of lipid peroxidation when plants suffer oxidative stress and it often serves as an indicator of the extent of membrane damage due to lipid peroxidation [23]. In this study, the leaf water content decreased during drought stress, and the MDA content of *R. soongorica* increased significantly after UV-B radiation and drought stress (Fig. 3A, B), which suggested that UV-B radiation and drought stress caused oxidative damage to *R. soongorica* and that membrane function was compromised. These results were in agreement with studies on soybean and wheat, which showed that MDA contents were increased by UV-B radiation and drought stress [25,26]. Meanwhile, Both UV-B and drought stress induced a decrease in the chlorophyll content of *R. soongorica* (Fig. 3C). It was reported that pigment contents reinforced the stability of membrane, and reductions in the pigment content may be because of the destruction of photosynthetic membranes by ROS [27].

In recent years, several genes encoding important enzymes, such as phenylalanine ammonia-lyase (PAL), F3H, dihydroflavonol 4-reductase (DFR), ANS (anthocyanin synthase) and F3′H, involved in the flavonoid biosynthetic pathway, have been found to be up-regulated in response to a number of different kinds of stress, such as salinity, UV-B irradiation, water deficits and nitrogen stress [14,15,28,29]. Zuker et al. [7] found modification of flower color and fragrance via antisense suppression of F3H, which suggested that the F3H were involved in the attraction of pollinators. In this study, the results showed that *RsF3H* was induced at the transcriptional level by UV-B radiation and drought stress (Fig. 4A). Similar results were observed in lettuce [14] that the transcript level of F3H increased under UV-B irradiation. This may be explained by the fact that flavonoid pathway genes can be controlled through UV-B photoreceptors which mediate plant responses to UV-B radiation [30]. Higher transcript levels of the flavonoid pathway genes: CHS, CHI and F3H, were also detected in potato after drought stress, supporting the hypothesis that the genes involved in the flavonoid pathway were induced by drought stress [18]. Moreover, the *RsF3H* protein activity was induced within 6 days and 4 days during UV-B radiation and drought stress treatments, respectively (Fig. 4B). These results were consistent with earlier investigations on activity of key enzymes in flavonoid pathway [31,32]. The increase in enzyme activities has been found to be attributable to increased transcription rates [26]. Pelletier et al. [33] also found that F3H and CHS expression caused the variations in their enzymes activity and our results confirmed this viewpoint. Therefore, *RsF3H* was both induced at the transcriptional and post-translational levels in order

to respond to UV-B and drought treatment in *R. soongorica*. As a consequence, more carbon-containing secondary metabolites, such as flavonoid, will be produced from primary products in plants.

In order to see whether the induction of *RsF3H* is accompanied by the accumulation of flavonoid, the total flavonoid and anthocyanin contents were investigated. UV-B radiation induced the accumulation of flavonoid, total phenols, and other UV-absorbing compounds in plants [16]. Flavonoid may play screening roles in the leaf by absorbing UV-B before it reaches to UV-sensitive targets such as chloroplasts and other organelles. In this study, the total flavonoid content decreased within 4 days and then showed an increasing trend under UV-B treatment (Fig. 4C). The UV-B radiation may have damaged the plant and resulted in metabolic disturbances initially [34]. After 4 days of adaptation, *R. soongorica* recovered possibly by increasing its resistance to UV-B radiation through the accumulation of flavonoid. The protective role for UV-B absorption is indicated by flavonoid deficient mutant in *Arabidopsis*, which showed hypersensitive to UV-B irradiation [35]. This mechanism is thought to be responsible for inhibiting the penetration of UV-B radiation into the leaf, and has been proved in previous studies in many other plants [16,36]. Flavonoid compounds are known to play significant roles in the resistance of plants to water deficit stress. At the same time, under the drought treatment, the total flavonoid content increased as the treatment time increased. Research in grape berries under drought stress found the same trend in flavonoid content [15]. It has been reported that flavonoid have a strong scavenging activity for ROS, including H_2O_2, O_2S^- and SOH radicals [17]. Anthocyanin, a class of flavonoid compound, has a higher level of hydroxylation. The higher levels of hydroxylation of flavonoid, the greater their anti-oxidative capacity [2]. In this study, the anthocyanin content increased under both UV-B radiation and drought treatment (Fig. 4D). This finding is consistent with the findings of von-Wettberg et al. [37], who found increases in the anthocyanin content under UV-B and drought stress. These results also support the hypothesis that plants accumulate more anthocyanin with a higher anti-oxidant property in order to resist the oxidative stress caused by UV-B radiation and drought treatments [2]. Several researches have demonstrated that flavonoid have the property of directly scavenging ROS [17]. To obtain more direct evidence, this study analyzed anti-oxidative ability by determining the inhibition of lipid oxidation of flavonoid. The result showed that the anti-oxidative ability of total flavonoid increased markedly as treatment time increased (Fig. 4E). These results were in agreement with Pietta [17], who argued that UV-B radiation and drought induced the proliferation of ROS and that flavonoid act as antioxidants by donating an electron to an oxidant in order to quench ROS.

In conclusion, the results suggested that flavonoid biosynthetic pathway may be involved in *R. soongorica* under UV-B radiation and drought stress. This research revealed that supplementary UV-B radiation and drought stress induced *RsF3H* expression and enzyme activity, as well as an increase in the production of anthocyanin content, which enhanced the antioxidant abilities of *R. soongorica* and helped it survive stressful conditions. More direct evidence on the physiological roles of *RsF3H* gene and the flavonoid biosynthetic pathway in response to abiotic stresses would be gained in our further studies.

4. Materials and methods

4.1. Plant materials and treatments

Seeds of *R. soongorica* were collected from the northern foothills of Lanzhou City, Gansu, China (36°17′N, 103°48′E, 1700–1900 m elevation) and were planted in individual 2-L plastic pots containing soil, in May 2009. The pots were placed in the experimental field at the Shapotou Desert Research & Experiment Station, Chinese Academy of Sciences. The regenerated plants were used for experiments in 2011.

Stress treatment started on August 2nd, 2011. Three treatment groups were established: UV-B radiation treatment, drought treatment and the control. According to the records of the Shapotou meteorological station, the maximum UV-B radiation intensity of the local natural environment is 2.5 W m^{-2}. For the UV-B radiation treatment, plants were exposed to natural radiation with a supplementary UV-B radiation of 0.5 W m^{-2} by hanging UV-B lamps (UVB-313, 40 W, Chenchen Lighting and Electronics Company, China) over the plants in the field for 10 days from 6:00 to 20:00. For the drought treatment group, two layers of thick plastic membrane and a removable rain shed were placed under, and over, individual pots in order to avoid the utilization of exogenous water. Before the drought treatment, the plants were fully irrigated and then dehydrated by not supplying water for 10 days. The control plants were exposed to light and rainfall, and were watered routinely during the entire experimental period. Each treatment had three replicates. Leaves from each treatment were collected every 2 days and then immediately stored in liquid nitrogen for later use.

4.2. Cloning of RsF3H cDNA from R. soongorica

The full length cDNA of *RsF3H* was obtained using the RACE method. A pair of degenerate primers: *RsF3H*-F (5′-TCC/AA/GGTTTGATAT/CGTCCGGT/CGGCAAG-3′) and *RsF3H*-R (5′-TTGCTCA TCTTCCTCCTG/ATACATCTC-3′) were designed and synthesized for amplifying the *RsF3H* core region. The 5′ end of *RsF3H* cDNA was amplified using the GeneRacer kit (Invitrogen, Carlsbad, USA) with *RsF3H*-5F (5′-ACGAGGACACTGACATGGA-3′) and *RsF3H*-5R (5′-CTATCTCTCGCCAATCTTGC-3′). The 3′-Full RACE core set Ver. 2.0 (Takara, Japan) was used to amplify the 3′ end of *RsF3H* cDNA with *RsF3H*-3F (5′-GTGCCTTTGTTGTTAACCTC-3′) and *RsF3H*-3R (5′-TACCGTCGTTCCACTAGTGATTT-3′). By aligning the sequences of the core region and the 5′ and 3′ RACE products, the full-length cDNA sequence of *RsF3H* was obtained. To confirm its authenticity, the open reading frame (ORF) sequence was subsequently amplified using PCR and a pair of primers: *RsF3H*-FF (5′-TATCCATTCCTGCAATTCTCA-3′, located before the start codon, ATG) and *RsF3H*-FR (5′-ACAAACCAA-GACAAGGACGTA-3′, located after the stop codon, TAA). The PCR product was then sequenced.

The obtained sequences were analyzed using the following website: www.ncbi.nlm.nih.gov. Alignment of deduced protein sequences was undertaken using DNAMAN (version 6.0.3.99). Several sequences of F3H downloaded from GenBank were used to construct an unrooted phylogenetic tree. The tree was constructed using the MEGA 5.1 program with the UPGMA method and evaluated by bootstrap analysis.

4.3. Determination of soil and water content

The soil water contents were measured using a mobile time domain reflectometry (TDR) probe (soil profiler PR2, Delta T Devices, UK). Meanwhile, plant leaf water content (%) was determined in freshly collected leaves and dried at 70 °C until constant weight. The leaf water content (LWC) of *R. soongorica* was calculated according to the following formula:

$$\mathrm{LWC}\ (\%) = \left[\left(W_f - W_d\right)\Big/ W_f\right] \times 100\%$$

where W_f and W_d are the fresh weight and dry weight, respectively.

4.4. Lipid peroxidation analysis

The level of lipid peroxidation content was determined as content of malonaldehyde (MDA) using the thiobarbituric acid (TBA) method [24]. 0.5 g leaves were homogenized in 10 ml of a solution containing 20% trichloroacetic acid and 0.5% 2-thiobarbituric acid. The mixture was heated at 95 °C for 30 min and then quickly placed to an ice bath. The cooled mixture was centrifuged at 5000 *g* for 10 min at room temperature, and the absorbance of the supernatant at 532 and 600 nm was recorded.

4.5. Chlorophylls content analysis

Total chlorophylls were determined using spectrophotometry method after extracted by 80% acetone as solvent [38].

4.6. Quantitative real-time RT-PCR (qRT-PCR) analysis

The RNA isolated from treated *R. soongorica* leaves was reverse transcribed using a PrimeScript RT reagent kit (Perfect Real Time) (Takara, Dalian, China). *RsF3H*-specific primers: *RsF3H*-QF (5′-GTTCAAGAATGCAGATCACCAAG-3′) and *RsF3H*-QR (5′-GGTTTCT CCCCTTCCCTTACA-3′) were designed and synthesized by Takara Biotechnology (Takara, Dalian, China). As an endogenous control, the primers: 18S-F (5′-ATGATAACTCGACGGATCGC-3′) and 18S-R (5′-CTTGGATGTGGTAGCCGTTT-3′) were used to amplify the 18S rRNA gene. The qRT-PCR was performed using the SYBR Premix Ex Taq (Takara, Dalian, China) in order to investigate the mRNA expression of *RsF3H*. The amplification protocol was carried out at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 62 °C for 60 s. Melt curve data were collected, starting at 70 °C and increasing in temperature at 0.2 °C increments by 120 cycles, for 15 s. The relative gene expression (*F*) was normalized against the housekeeping gene according to the formula:

$$F = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{housekeeping}})^{\Delta Ct_{\text{housekeeping}}(\text{control}-\text{sample})}}$$

where $E = 1.09$, which represents the amplification efficiency and is obtained according to standard curve for target gene.

4.7. Determination of RsF3H activity

The extraction and activity assays of the *RsF3H* enzymes were performed as described by Beno-Moualem et al. [39]. 0.1 g fresh leaves were grinded in a mortar with liquid nitrogen, and homogenized with 1 ml of 0.1 M Tris–HCl (pH 7.4), and 0.2 g polyvinyl pyrrolidone. The extract was centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatant was used as an enzyme source. *RsF3H* activity was determined in a 0.6 ml of reaction mixture containing 50 mM Tris–HCl (pH 7.4), 0.015 mM naringenin (Sigma, St Louis, USA), 5 mM NADPH, and 100 μg enzyme extract. The reaction was incubated in a water bath at 37 °C for 2 h and then terminated with adding 50 μl of 5 M HCl. And the naringenin which is the enzyme substrate was extracted three times with 1 ml ethyl acetate. The upper layer was transferred to a new tube and evaporated under N_2. The dry extracts were dissolved with 0.2 ml of a mixture of methanol/doubled distilled water/acetic acid (55/44/1, v/v/v).

Naringenin was quantified by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Agilent, USA) and detection at 320 nm. In the HPLC analysis experiment, a 10 μl extract was injected onto a 2.1 mm × 30 mm C-18 column. The column was maintained at 20 °C and elution was performed with ethanol/water (1/1, v/v) at a flow speed of 0.2 ml min^{-1}. The mass spectra were recorded using ESI in the negative mode with gas spray at 10 psi. The activity is given as the disappearance of naringenin.

4.8. Determination of total flavonoid, anthocyanin content and antioxidant ability

The extraction of total flavonoid was conducted according to the method of Quettier-Deleu et al. [40]. Flavonoid were extracted by 75% ethanol after grinding of fresh leaves in cold mortar and then transferred to tubes for extraction at 75 °C for 2 h. Then the filtrate was evaporated on a rotary evaporator at 60 °C and the remaining residue was dissolved in 1 ml methanol. The obtained extract was used to determine antioxidant activity and the content of total flavonoid and anthocyanin.

Total flavonoid contents were quantified by the aluminum calorimetric method [38] using lutein for a standard. The reaction mixture contained 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum calorimetric, 0.1 ml of 1 M potassium acetate, 2.8 ml of distilled water, and 0.5 ml flavonoid extract. The mixture was incubated for 30 min at 25 °C. The content of total flavonoid was measured using a spectrophotometer at 510 nm. The absorbance of flavonoid extract at 530 was measured to quantify the amount of anthocyanin [41].

The ability of flavonoid extracts to prevent oxidation of linoleic acid to MDA was determined as previously described by Aqil et al. [42] with modification. A total of 0.38 mM linoleic acid was oxidized by adding 0.33 mM H_2O_2 in the presence of flavonoid extracts at room temperature for 5 h. The MDA contents that the reaction produced were measured by TBA method as described in Section 2.3.

4.9. Statistical analysis

Each experiment was performed at least in triplicate and all data were presented as means ± the standard deviation. Statistical analyses were performed using a one-way ANOVA. Duncan's test was used to distinguish differences between mean values. $P < 0.05$ was considered to be statistically significant.

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References

[1] B. Winkel-Shirley, Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology, Plant Physiol. 126 (2001) 485–493.
[2] B. Winkel-Shirley, Biosynthesis of flavonoids and effects of stress, Curr. Opin. Plant Biol. 5 (2002) 218–223.
[3] B. Winkel-Shirley, W.L. Kubasek, G. Storz, E. Bruggemann, M. Koomneef, F.M. Ausubel, H.M. Goodman, Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis, Plant J. 8 (1995) 659–671.
[4] S. Hassan, U. Mathesius, The role of flavonoids in root-rhizosphere signaling: opportunities and challenges for improving plant-microbe interactions, J. Exp. Bot. 63 (2012) 3429–3444.
[5] F. Sparvoli, C. Martin, A. Scienza, G. Gavazzi, C. Tonelli, Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.), Plant Mol. Biol. 24 (1994) 743–755.
[6] B. Yu, D. Zhang, C.H. Huang, M.J. Qian, X.Y. Zheng, Y.W. Teng, J. Su, Q. Shu, Isolation of anthocyanin biosynthetic genes in red Chinese sand pear (*Pyrus pyrifolia Nakai*) and their expression as affected by organ/tissue, cultivar, bagging and fruit side, Sci. Hortic. (Amsterdam) 136 (2012) 29–37.

[7] A. Zuker, T. Tzfira, H. Ben-Meir, M. Ovadis, E. Shklarman, H. Itzhaki, G. Forkmann, S. Martens, I. Neta-Sharir, D. Weiss, A. Vainstein, Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase, Mol. Breed. 9 (2002) 33–41.
[8] M. Nishihara, T. Nakatsuka, S. Yamamura, Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene, FEBS Lett. 579 (2005) 6074–6078.
[9] Y. Ueyama, K. Suzuki, M. Fukuchi-Mizutani, Y. Fukui, K. Miyazaki, H. Ohkawa, T. Kusumi, Y. Tanaka, Molecular and biochemical characterization of torenia flavonoid 3′-hydroxylase and flavone synthase II and modification of flower color by modulating the expression of these genes, Plant Sci. 163 (2002) 253–263.
[10] E. Fukusaki, K. Kawasaki, S. Kajiyama, C. An, K. Suzuki, Y. Tanaka, A. Kobayashi, Flower color modulations of *Torenia hybrid* by down-regulation of chalcone synthase genes with RNA interference, J. Biotechnol. 111 (2004) 229–240.
[11] A. Nakatsuka, D. Mizuta, Y. Kii, I. Miyajima, N. Kobayashi, Isolation and expression analysis of flavonoid biosynthesis genes in evergreen azalea, Sci. Hortic. (Amsterdam) 118 (2008) 314–320.
[12] R. Lukačin, L. Britsch, Identification of strictly conserved histidine and arginine residues as part of the active site in *Petunia hybrida* flavanone 3ß-hydroxylase, Eur. J. Biochem. 249 (1997) 748–757.
[13] R.A. Dixon, N.L. Paiva, Stress-induced phenylpropanoid metabolism, Plant Cell 7 (1995) 1085–1097.
[14] J.S. Park, M.G. Choung, J.B. Kim, B.S. Hahn, J.B. Kim, S.C. Bae, K.H. Roh, Y.H. Kim, C.I. Cheon, M.K. Sung, K.J. Cho, Genes up-regulated during red coloration in UV-B irradiated lettuce leaves, Plant Cell Rep. 26 (2007) 507–516.
[15] S.D. Castellarin, M.A. Matthews, G.D. Gaspero, G.A. Gambetta, Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries, Planta 227 (2007) 101–112.
[16] P. Burchard, W. Bilger, G. Weissenböck, Contribution of hydroxycinnamates and flavonoids to epidermal shielding of UV-A and UV-B radiation in developing rye primary leaves as assessed by ultraviolet-induced chlorophyll fluorescence measurements, Plant Cell Environ. 23 (2000) 1373–1380.
[17] P.G. Pietta, Flavonoids as antioxidants, J. Nat. Prod. 63 (2000) 1035–1042.
[18] C.M. André, R. Schafleitner, S. Legay, I. Lefèvre, C.A.A. Aliaga, G. Nomberto, L. Hoffmann, J.F. Hausman, Y. Larondelle, D. Evers, Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress, Phytochemistry 70 (2009) 1107–1116.
[19] L.O. Morales, R. Tegelberg, M. Brosché, M. Keinänen, A. Lindfors, P.J. Aphalo, Effects of solar UV-A and UV-B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves, Tree Physiol. 30 (2010) 923–934.
[20] S.K. Lenka, A. Katiyar, V. Chinnusamy, K.C. Bansal, Comparative analysis of drought-responsive transcriptome in Indica rice genotypes with contrasting drought tolerance, Plant Biotechnol. J. 9 (2011) 315–327.
[21] J. Bai, C.M. Gong, K. Chen, H.M. Kang, G. Wang, Examination of antioxidative system's responses in the different phases of drought stress and during recovery in desert plant *Reaumuria soongorica* (Pall.) Maxim, J. Plant Boil. 52 (2009) 417–425.
[22] Y.B. Liu, T.G. Zhang, X.R. Li, G. Wang, Protective mechanism of desiccation tolerance in *Reaumuria soongorica*: leaf abscission and sucrose accumulation in the stem, Sci. China Ser. C 50 (2007) 15–21.
[23] R.S. Dhindsa, W. Matowe, Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation, J. Exp. Bot. 32 (1981) 79–91.
[24] K.V. Madhava-Rao, T.V.S. Sresty, Antioxidative parameters in the seedlings of pigeon pea (*Cajanus cajan* (L.) Millspaugh) in response to Zn and Ni stresses, Plant Sci. 157 (2000) 113–128.
[25] X.M. Li, L.H. Zhang, Y.Y. Li, L.J. Ma, N. Bu, C.Y. Ma, Changes in photosynthesis, antioxidant enzymes and lipid peroxidation in soybean seedlings exposed to UV-B radiation and/or Cd, Plant Soil 352 (2012) 377–387.
[26] Y. Yang, F. He, L. Yu, X.H. Chen, J. Lei, J.X. Ji, Influence of drought on oxidative stress and flavonoid production in cell suspension culture of *Glycyrrhiza inflata Bata*, Z. Naturforsch. C 62 (2007) 410–416.
[27] N.I. Elsheery, K.F. Cao, Gas exchange, chlorophyll fluorescence, and osmotic adjustment in two mango cultivars under drought stress, Acta Physiol. Plant 30 (2008) 769–777.
[28] H. Walia, C. Wilson, P. Condamine, X. Liu, A.M. Ismail, L. Zeng, S.I. Wanamaker, J. Mandal, J. Xu, X.P. Cui, T.J. Close, Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage, Plant Physiol. 139 (2005) 822–835.
[29] Y.P. Han, S. Vimolmangkang, R.E. Soria-Guerra, S. Rosales-Mendoza, D. Zheng, A.V. Lygin, S.S. Korban, Ectopic expression of apple F3′H genes contributes to anthocyanin accumulation in the *Arabidopsis* tt7 mutant grown under nitrogen stress, Plant Physiol. 153 (2010) 806–820.
[30] P.V. Demkura, C.L. Ballare, UVR8 mediates UV-B-induced *Arabidopsis* defense responses against *Botrytis cinerea* by controlling sinapate accumulation, Mol. Plant 5 (2012) 116–126.
[31] Y. Ban, C. Honda, H. Bessho, X.M. Pang, T. Moriguchi, Suppression subtractive hybridization identifies genes induced in response to UV-B irradiation in apple skin: isolation of a putative UDP-glucose 4-epimerase, J. Exp. Bot. 58 (2007) 1825–1834.
[32] X.Y. Shen, S. Martens, M.L. Chen, D.F. Li, J.L. Dong, T. Wang, Cloning and characterization of functional flavanone-3ß-hydroxylase gene from *Medicago truncatula*, Mol. Biol. Rep. 37 (2010) 3283–3289.
[33] M.K. Pelletier, I.E. Burbulis, B. Winkel-Shirley, Disruption of specific flavonoid genes enhances the accumulation of flavonoids enzymes and end-products in *Arabidopsis* seedlings, Plant Mol. Biol. 40 (1999) 45–54.
[34] Č. Čejka, J. Plášteník, J. Širc, T. Ardan, J. Michálek, B. Brůnová, J. Čejková, Changes of corneal optical properties after UVB irradiation investigated spectrophotometrically, Physiol. Res. 59 (2010) 591–597.
[35] V. Mishara, P. Mishra, G. Srivastava, S.M. Prasad, Effect of dimethoate and UV-B irradiation on the response of antioxidant defense systems in cowpea (*Vigna unfuiculata* L.) seedlings, Pestic. Biochem. Phys. 100 (2011) 118–123.
[36] G. Agati, M. Tattini, Multiple functional roles of flavonoids in photoprotection, New Phytol. 186 (2010) 786–793.
[37] E.J. von-Wettberg, M.L. Stanton, J.B. Whittall, How anthocyanin mutants respond to stress: the need to distinguish between stress tolerance and maximal vigour, Evol. Ecol. Res. 12 (2010) 457–476.
[38] E.W. Chappelle, M.S. Kim, J.E. McMurtrey III, Ratio analysis of reflectance spectra (RARS): an algorithm for the remote estimation of the concentrations of chlorophyll a, chlorophyll b, and carotenoids in soybean leaves, Remote Sens. Environ. 39 (1992) 239–247.
[39] D. Beno-Moualem, Y. Vinokur, D. Prusky, Cytokinins increase epicatechin content and fungal decay resistance in Avocado fruits, J. Plant Growth Regul. 20 (2001) 95–100.
[40] C. Quettier-Deleu, B. Gressier, J. Vasseur, T. Dine, C. Brunet, M. Luyckx, M. Cazin, J.C. Cazin, F. Bailleul, F. Trotin, Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour, J. Ethnopharmacol. 72 (2000) 35–42.
[41] M.F.A. Bakar, M. Mohamed, A. Rahmat, J. Fry, Phytochemicals and antioxidant activity of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*), Food Chem. 113 (2009) 479–483.
[42] F. Aqil, I. Ahmad, Z. Mehmood, Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants, Turk. J. Biol. 301 (2006) 77–183.