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Gene expression and flavonol biosynthesis are induced by ultraviolet-B and salt stresses in *Reaumuria trigyna*

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Abstract

In plants, flavonoids play roles not only in development, but also in responses to biotic and abiotic stresses. We analyzed the transcriptome data of NaCl-treated *Reaumuria trigyna*, a small, highly haloduric desert shrub, focusing on the flavonoid biosynthetic pathway. We identified 118 unigenes annotated as genes encoding enzymes related to flavonoid biosynthesis, 68 of which were differentially expressed under NaCl treatment (39 upregulated, 29 downregulated). Of the 118 annotated unigenes, 47 were annotated as members of families related to the flavonol biosynthetic pathway (*e.g.*, *F3H*, *FLS*, and *OMT*). Of those 47 genes, about 70 % (32 unigenes) were upregulated under NaCl treatment. Experiments were conducted to monitor changes in gene expression and accumulation of total polyphenols, total flavonols, and antioxidant capacity under NaCl and ultraviolet-B (UV-B) radiation treatments. The expressions of genes related to the flavonol biosynthesis pathway (*RtC4H*, *RtCHS*, *RtF3H3*, *RtFLS1*, *RtFLS2*, *RtF3′5′H*, *RtF3′H*, *RtOMT*, and *RtMYBF1*) increased under NaCl and UV-B treatments. Treatments with NaCl and UV-B also increased the total flavonols content and antioxidant activity. The content of several flavonols including rutin, hyperoside, isorhamnetin-3-*O*-neohespeidoside, and myricetin increased in response to NaCl and UV-B stresses. Overall, our results show that the expression of genes related to flavonol biosynthesis as well as flavonol content increased in *R. trigyna* under NaCl and UV-B stresses.

Additional key words: antioxidants, differentially expressed genes, flavonoids, reactive oxygen species, transcriptome.

Introduction

Flavonoids are a diverse group of plant secondary metabolites that are widely distributed throughout the plant kingdom. In terrestrial plants, nine subclasses of flavonoids, *i.e.*, chalcones, flavones, flavonols, dihydroflavonols, flavandiols, anthocyanins, proanthocyanidins, isoflavonoids, and aurones have been categorized based on their chemical structures (Winkel-Shirley 2001, Martens *et al.* 2010). They are of a great pharmaceutical interest because they have health-promoting effects, including antioxidant, anticancer, anti-inflammatory, cardioprotective, and neuroprotective effects on humans and animals (Harborne and Williams 2000, Misra *et al.* 2010). In plant tissues, flavonoids function as pigments and seed coat components; act as hormone-like signals in pollen tube development, reproduction, and plant-microbe interactions; play protective roles against pests and pathogens; and regulate auxin transport during plant growth and development (Simmonds 2003, Kobayashi *et al.* 2004, Koes *et al.* 2005, Peer and Murphy 2007, Stracke *et al.* 2007, Buer *et al.* 2010, Misra *et al.* 2010, Hassan and Mathesius 2012, Pandey *et al.* 2012). Flavonoids are also produced as a protective response to environmental stresses. Flavonoid biosynthesis is upregulated under a wide range of abiotic stresses. For example, in *Arabidopsis thaliana*, phosphate, nitrogen, high irradiance, temperature, and drought stresses affect the expressions of genes related to the flavonoid synthesis pathway (Rowan *et al.* 2009, Catalá *et al.* 2011, Lei *et al.* 2011, Nakabayashi *et al.* 2014). Ultraviolet (UV) radiation is one of the strongest inducers of flavonoid accumulation, and many studies have shown that flavonoid synthesis can protect plants from UV-induced damage (Landry *et al.* 1995, Jansen *et al.* 2008, Wang *et al.* 2011, Hectors *et al.* 2014). For example,

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Abbreviations: C4H - cinnamate 4-hydroxylase; CHI - chalcone isomerase; CHS - chalcone synthase; 4CL-4 - coumaroyl-CoA ligase; DEGs - differentially expressed genes; DFR - dihydroflavonol reductase; DPPH - 2,2-diphenyl-1-picrylhydrazyl; ECOD-7 - ethoxycoumarin O-deethylase, F3H - flavanone 3-hydroxylase; F3′H - flavonoid 3′-hydroxylase; F3′5′H - flavonoid 3′,5′-hydroxylase; FLS - flavonol synthase; GST - glutathione S-transferase; MS - Murashige and Skoog; OMT - *O*-methyltransferase; PAL - phenylalanine ammonia lyase; ROS - reactive oxygen species; UV-B - ultraviolet-B radiation.

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the UV-induced flavonoid accumulation in *Betula pendula* (Zhang *et al.* 2011) and *Asparagus officinalis* (Eichholz *et al.* 2012) appear to protect plants from stress-induced reactive oxygen species (ROS). Emiliani *et al.* (2013) show that *Arabidopsis* plants over-accumulating flavonols showed reduced ultraviolet-B (UV-B) damage with lower DNA damage and lipid peroxidation.

The flavonoid biosynthesis pathway has been extensively studied in several model plants including *Antirrhinum majus*, *Petunia hybrida*, and *Arabidopsis thaliana*. Many of the genes encoding biosynthetic enzymes and transcription factors involved in the pathway are well documented (Beld *et al.* 1989, Routaboul *et al.* 2006, Hichri *et al.* 2011). The transcriptional regulation of structural genes tightly controls flavonoid biosynthesis during plant development and in response to biotic and abiotic stresses (Lepiniec *et al.* 2006). In grape berries, the key flavonol biosynthesis gene *flavonol synthase 1* (*FLS1*) was shown to be upregulated by UV-B, leading to increased flavonol content in the skin. Changes in flavonol and anthocyanin composition could be largely explained by the transcript amounts of *flavonoid 3'-hydroxylase* (*F3'H*), *flavonoid 3',5'-hydroxylase* (*F3'5'H*), and *O-methyltransferase* (*OMT*) (Martínez-Lüscher *et al.* 2014). In wheat, drought stress increased the expression of *chalcone synthase* (*CHS*), *flavanone 3-hydroxylase* (*F3H*), *FLS*, and *dihydroflavonol reductase* (*DFR*), resulting in increased flavonoid accumulation (Castellarin *et al.* 2007). To date, the effects of abiotic stress on the biosynthesis and degradation of flavonoids have been investigated mainly in crop and model plants. Comparatively few studies have focused on flavonoid biosynthesis in wild stress-resistant plants in response to diverse environmental cues.

Plants that grow in dry climate are likely to have evolved physiological and molecular regulation mechanisms related to stress resistance. It is useful to explore these mechanisms of wild plants that have evolved in such environments. *Reaumuria trigyna*, an endangered dicotyledonous shrub with the features of a halophyte, is endemic in a salinized desert in Inner Mongolia. The area is characterized by high soil salinity, drought (annual average precipitation is 141 - 302 mm), and extreme temperatures ranging from -36.6 to 68.5 °C (Dang *et al.* 2013). Several studies have analyzed the physiological and molecular responses of *R. trigyna* to stress environments, its osmotic regulation (Xue and Wang 2008), and the expression of stress-inducible genes under salinity stress (Dang *et al.* 2013). However, little is known about the functional effects of secondary metabolic pathways in *R. trigyna* under various stress conditions.

In this study, we analyzed the transcriptome data of *R. trigyna*, focusing on the flavonoid synthesis pathway to identify differentially expressed genes (DEGs) under NaCl treatment. To further understand the regulation of flavonol biosynthesis, the transcription of key genes and flavonol composition were evaluated after treatments with NaCl at different concentrations. UV radiation is one of the strongest inducers of flavonoid biosynthesis, and *R. trigyna* grows at an altitude of 1500 - 2100 m a.s.l. with extraordinarily high UV-B radiation. Therefore, the expressions of genes encoding key enzymes in the flavonol biosynthesis pathway, as well as flavonol content, were also analyzed under different UV-B radiation.

Materials and methods

Plants, growth conditions, and treatments: Seeds of *Reaumuria trigyna* Maxim. (family *Tamaricaceae*) were collected from the eastern Alxa-Western Ordos area in Inner Mongolia. Seeds were sterilized in 10 % (m/v) sodium hypochlorite for 15 min, and then rinsed three times with sterilized double-distilled water. Seeds were germinated on Murashige and Skoog (MS) medium in the dark for 72 h, and then grown on the same medium at a temperature of 25 °C, a relative humidity of 70 %, and a 16-h photoperiod with irradiance of 54 μmol m^{-2} s^{-1} for 20 d. Seedlings were grown to at least 10 cm before being transferred to MS liquid medium and grown for 3 weeks.

Healthy seedlings of similar sizes were subjected to either NaCl or UV-B stress. For the NaCl treatment, seedlings were grown for 10 d in MS liquid medium supplemented with NaCl at a final concentrations of 100, 300, or 500 mM. For the UV-B treatments, seedlings were treated with white light supplemented with UV-B radiation (7.5 - 12.5 μmol m^{-2} s^{-1}) for 12 h, followed by an 8-h dark period. This alternation continued for a total of 52 h, and samples were collected after 12, 24, and 36 h of UV-B treatment. The UV-B radiation was supplied by UV-B lamps (15 W, *G15T8E*, *Sanyo*, Osaka, Japan), which emit UV radiation between 280 and 360 nm, with a peak at 306 nm. Therefore, the UV-B treatments included some UV-A radiation (315 - 360 nm). The distance between the UV-B lamps and the plants was about 25 cm. Seedlings were harvested after the NaCl and UV-B treatments. Control plants that were not subjected to UV-B and NaCl treatments were harvested at the same time.

RNA extraction and first-strand cDNA synthesis: Analyses of the abundance of gene transcripts related to flavonoid biosynthesis were performed as described previously (Dang *et al.* 2013). Frozen samples were ground to a fine powder in liquid nitrogen in a precooled mortar and pestle. Then, RNA was extracted using a *MiniBEST* plant RNA extraction kit (*Takara*, Kyoto, Japan), according to the manufacturer's instructions. Before reverse transcription, the quality of the extracted RNA was checked by agarose gel electrophoresis and verified using an UV spectrometer (absorbance ratios A_{260}/A_{230} 2.0 - 2.2 and $A_{260}/A_{280} > 2.0$). First-strand cDNA was synthesized using *PrimeScript™ RT Master Mix* (*Takara*), according to the manufacturer's instructions.

***KEGG* pathway analysis of flavonoid biosynthesis and transcription factor identification:** Genes in the flavonoid biosynthesis pathway were identified by *Illumina* sequencing using a functional annotation file based on the candidate gene name. We used a basic local alignment search tool (*BLAST*) and the National Center for Biotechnology Information (*NCBI*) GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). We also performed a *BLAST* search for *R. trigyna* transcription factors in the plant transcription factor database (http://planttfdb.cbi.pku.edu.cn/blast.php).

Gene expression analysis by quantitative PCR: Based on the sequences of six unigenes in the flavonoid pathway, real-time PCR primers were designed by the Beijing Genomics Institute (BGI, Beijing, China; Table 1 Suppl). *β-Actin* was used as the internal control gene. The qPCR analyses were performed on the *Qiagen Rotor-gene Q* real-time PCR platform (*Qiagen*, Hilden, Germany) using a *KAPA SYBR® FAST* universal qPCR kit (*Kapa Biosystems*, Boston, MA, USA) to detect transcript abundance. The thermal cycling conditions were as follows: first denaturation at 95 °C for 30 s; then 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 55 °C for 30 s. The relative expressions of the selected unigenes were calculated using the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method. All reactions were performed with three replicates, and data were analyzed using *Rotor-gene Q* series software.

Measurements of total polyphenol content: Total phenolics were extracted from freeze-dried samples (50 mg) in 1 cm^3 of 80 % (v/v) ethanol solution at 4 °C for 2 h in the dark. The polyphenol extracts of *A. thaliana* and *R. trigyna* were named AtF and RtF, respectively. Then, HCl was added to a final concentration of 0.1 M and the mixture was incubated at 98 °C for 1 h to hydrolyze any conjugate forms of flavonoids (Bulgantuya *et al.* 2015). After hydrolysis, samples were evaporated to dryness and then resuspended in 1 cm^3 of methanol. Gallic acid diluted with ethanol was used to produce the calibration curve (y = 0.0135 x - 7E-06, R^2 = 0.99956). A 0.1-cm^3 aliquot of the standard solution or plant extract was mixed with 2.4 cm^3 of diluted Folin-Ciocalteu phenol reagent (1:1 with water). The reaction mixture was incubated at room temperature for 5 min before adding 2.5 cm^3 of Na_2CO_3 (20 %, m/v). The mixture was incubated for 1 h at room temperature and then the absorbance at 710 nm was measured using a *SpectraMax® i3* plate reader (*Molecular Devices*, Sunnyvale, CA, USA). The total polyphenol content of each sample was calculated as follows: total polyphenols [mg g^{-1}] = y × 50 × 1/0.05, where y is content of gallic acid in total polyphenols according to the calibration curve (Pandey *et al.* 2014).

Measurements of total flavonol content: Total flavonols were extracted in an 80 % (v/v) methanol solution at 4 °C for 12 h (50 mg sample +1 cm^3 of methanol solution). The flavonol extracts of *A. thaliana* and *R. trigyna* were named AtP and RtP, respectively. Then, HCl was added to a final concentration of 0.1 M and the mixture was incubated at 98 °C for 1 h to hydrolyze any conjugate forms of flavonoids (Bulgantuya *et al.* 2015). After hydrolysis, samples were evaporated to dryness and resuspended in 1 cm^3 of methanol. Quercetin diluted with ethanol was used to make the calibration curve (y = 0.0377 x + 0.001, R^2 = 0.99758). A 0.5-cm^3 aliquot of the standard solution or plant extract was mixed with 1.5 cm^3 of 95 % (v/v) methanol, 2.8 cm^3 of double distilled H_2O, 0.1 cm^3 of 10 % (m/v) $AlCl_3$, and 1 M potassium acetate. After 30 min incubation at room temperature, absorbance was recorded at 415 nm using a *SpectraMax® i3* plate reader. The total flavonol content of each sample was calculated as follows: total flavonols [mg g^{-1}] = y × 10 × 1/0.05, where y is content of quercetin in total flavonols according to calibration curve (Pandey *et al.* 2014).

Antioxidant assay: The free radical-scavenging ability of plant extracts was assayed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide spectrophotometric methods. The DPPH-scavenging activity was measured according to the method of Sanchez-Moreno (2002) with some modifications. A 2.8-cm^3 aliquot of a methanolic solution of DPPH (0.1 mM) was mixed with 0.2 cm^3 of extract and incubated in the dark for 30 min. The absorbance of the mixture was measured at 517 nm. Ascorbic acid (vitamin C) was used as the positive control. The radical-scavenging activities of the tested samples were evaluated by comparison with a control containing 2.8 cm^3 of DPPH solution and 200 cm^3 of methanol. The antioxidant activity of each sample was calculated according to the following formula: antioxidant activity [%] = [(Ac - As)/Ac] × 100, where Ac = is the absorbance of the reaction mixture with methanol and As = is the absorbance of the reaction mixture with plant extract. The IC_{50} is defined as the amount of plant extract [μg cm^{-3}] required to decrease DPPH absorbance at 517 nm to one-half of its original absorbance (Naik *et al.* 2003). H_2O_2-scavenging activity was measured according to the method of Liu (Liu 2005). The 10-cm^3 reaction mixture consisted of 1 cm^3 of plant extract (1 cm^3 methanol as control), 3 cm^3 of 2 mM $FeSO_4$, 3 cm^3 of 6 mM salicylic acid, and 3 cm^3 of 1 mM H_2O_2. The reaction mixture was incubated for 1 h at 37 °C and its absorbance was measured at 517 nm. The H_2O_2-scavenging activity of each sample was calculated using the same equation as mentioned above.

Qualitative and quantitative analysis of flavonols: Freeze-dried samples were extracted in a 90 % (v/v) methanol solution (0.5 g sample + 10 cm^3 of methanol) using sonication at 4 °C for 40 min. The mixture was centrifuged at 13 000 *g* for 10 min and passed through a 0.22-μm filter, after which the volume was adjusted to 2 cm^3 with methanol solution. Rutin, hyperoside, myricetin, dihydromyricetin, tixifolin, quercetin, isorhamnetin-3-*O*-neohespeidoside, isorhamnetin, and kaempferide (*MUST*, Chengdu, China) standard solutions were prepared

by successive dilution of stock solutions (Table 2 Suppl). All samples were analyzed using an *Agilent 1290* ultra-performance liquid chromatograph and an *Agilent 6460* triple quadrupole mass spectrometer (*UPLC-QQQ-MS*) (*Agilent Technologies*, Singapore) with an *Agilent Eclipse Plus* C 18 column (50 × 2.1 mm i.d.; 1.8 μm particle size) at 30 °C. The mobile phase was 0.1 % (m/v) formic acid in H_2O (solvent A) and MeOH (solvent B). The elution program was as follows: 0.5 min, 10 % B; 1.0 min, 20 %; 3.0 min, 32 %; 5.5 min, 35 %; 7.8 min, 40 %; 8.5 min, 75 %; 9.5 min, 76 %; 9.6 min, 100 %; 12 min, 100 %, then post time 1 min. The injection volume was 2 mm^3 and the flow rate was 0.4 cm^3 min^{-1}. Multiple reaction monitoring was used for detection and qualitative analysis in the negative ion mode. The ion source parameters were set as follows: capillary, 4 000 V; gas temperature of 350 °C; gas flow of 11 dm^3 min^{-1}; nebulizer of 45 psi. All experiments were repeated three times.

Statistics: The experiments were repeated three times independently. Data were analyzed with the *SPSS* software using one-way analysis of variance (*ANOVA*). The significance of differences between treatments was analyzed by Tukey's test.

Results

In a previous study that analyzed transcriptomic data using *KEGG* enrichment analysis, an unexpected finding was that the flavonoid biosynthesis pathways were very active under NaCl stress (Dang *et al.* 2013). However, specific genes were not analyzed in that study. In this study, we analyzed the flavonoid synthesis pathway in the transcriptome data of *R. trigyna* under NaCl stress. In total, 118 unigenes encoding enzymes in the flavonoid biosynthesis pathway were identified, of which 68 were DEGs with 39 upregulated and 29 downregulated (Fig. 1). Within the general polyphenolic pathway, 11, 9, and 23 unigenes were identified, belonging to *phenylalanine ammonia-lyase* (*PAL*), *cinnamate 4-hydroxylase* (*C4H*), and *4-coumaroyl-CoA ligase* (*4CL*) gene families, respectively. An additional 11 unigenes were annotated as *CHS* and 6 as *chalcone isomerase* (*CHI*). Six unigenes belonged to DFR and LDOX gene families, which are involved in anthocyanin biosynthesis. Five

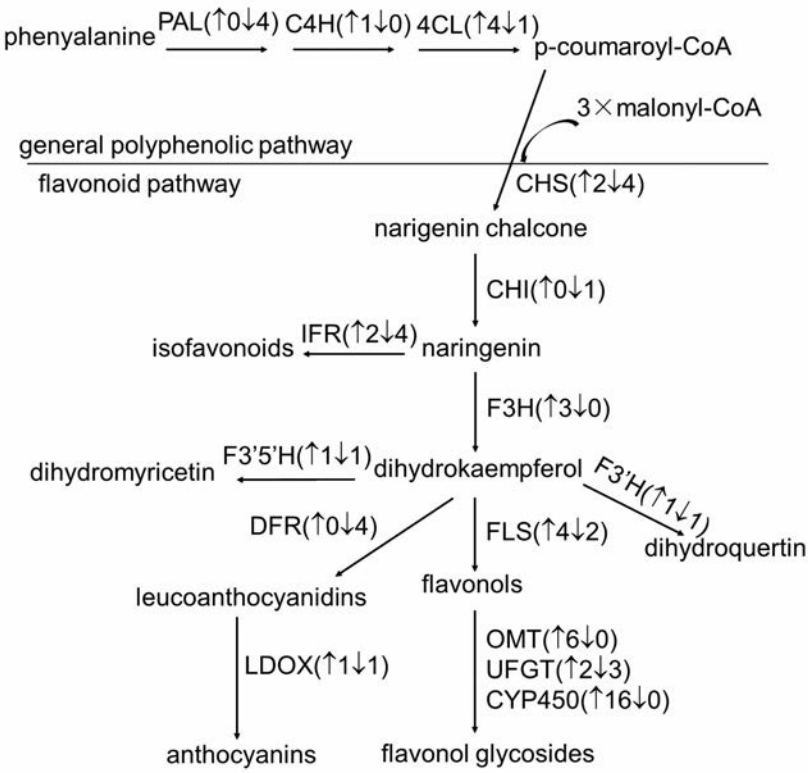

Fig. 1. Several differentially expressed genes (DEGs) were annotated with the same gene name in the flavonoid pathway. ↑indicates number of upregulated DEGs, ↓indicates number of downregulated DEGs. PAL - phenylalanine ammonia lyase; C4H - cinnamate 4-hydroxylase; 4CL -. 4-coumaroyl CoA ligase; CHS - chalcone synthase; CHI - chalcone isomerase; F3H - flavanone-3-hydroxylase; FLS - flavonol synthase; F3'H - flavonoid-3′-hydroxylase; F3′5′H - flavonoid-3′5′-hydroxylase; FNS - flavone synthase; IFS - isoflavone synthase; 12′H, isoflavone 2′-hydroxylase; IFR - isoflavone reductase; IOMT - isoflavone O-methyltransferase; DFR - dihydroflavonol 4-reductase; LDOX - leucoanthocyanidin dioxygenase; UFGT - UDP - flavonoid glucosyltransferase; CYP450 - cytochrome P_{450}; OMT - O-methyltransferase.

unigenes (all downregulated DEGs) encoded isoflavone reductase (*IFR*), a key enzyme in isoflavonoid biosynthesis. The other 47 unigenes belonged to other families: *F3H*, *F3'H*, *F3'5'H*, *FLS*, *OMT*, *UFGT*, and some types of *CYP450*, *e.g.*, *7-ethoxycoumarin O-deethylase* (*ECOD*) and *glutathione S-transferase* (*GST*). These families are also related to the flavonol biosynthesis pathway. Of these 47 unigenes in other families, about 70 % (32 unigenes) were upregulated DEGs under NaCl treatment (Table 3 Suppl and Table 5 Suppl). A total of 512 unigenes were annotated as transcription regulators in four families: *MYB*, *WD40*, *bHLH*, and *WRKY*. We identified 13 transcription factors that were putatively related to flavonoid biosynthesis in *R. trigyna*. These unigenes showed 70 - 80 % similarity to corresponding transcription factors that are known to play important roles in regulating the flavonoid biosynthesis pathway in *Vitis vinifera*, *Arabidopsis thaliana*, and *Catharanthus roseus* (Table 4 Suppl).

Based on the results of transcriptome analysis, we

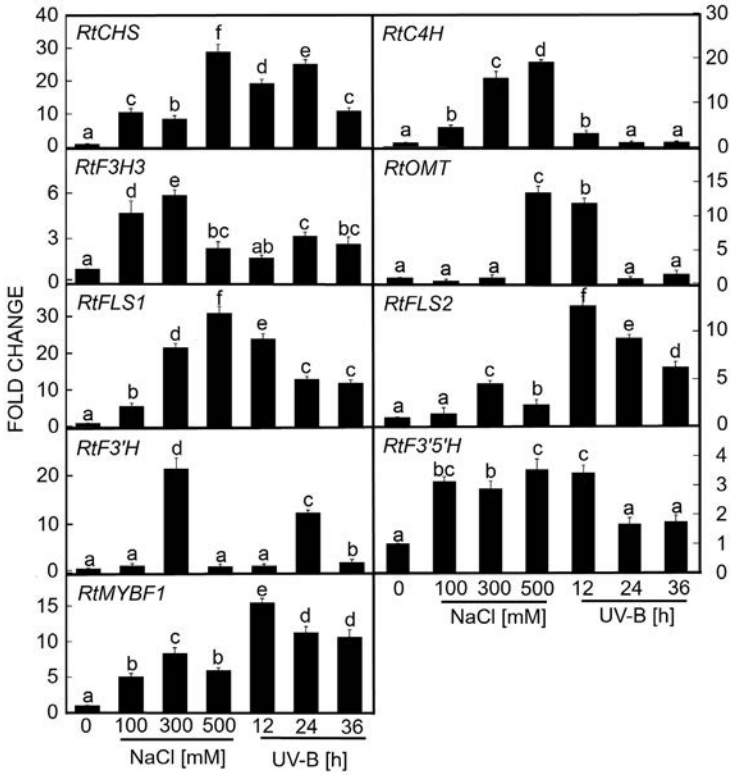

Fig. 2. Expression analysis of nine differentially expressed genes (DEGs) of the flavonol synthesis pathway under NaCl or UV-B stress in *Reaumuria trigyna.* Fold change represents the relative fold-increase in expression as compared to respective control. Control was set to 1. Means ± SDs (n = 3). Different letters indicate significant differences from control (P < 0.05).

selected nine DEGs (*RtC4H*, *RtCHS*, *RtF3H3*, *RtFLS1*, *RtFLS2*, *RtF3'5'H*, *RtF3'H*, *RtOMT*, and the transcription factor *RtMYBF1*) showing high expression for further analysis (Fig. 2). The expressions of these genes increased under NaCl stress and UV-B stress. The transcript amounts of *RtC4H*, *RtF3'5'H*, *RtCHS*, *RtOMT*, and *RtFLS1* increased gradually as the NaCl concentration increased from 100 mM to 500 mM. The expression of *RtF3H3*, *RtFLS2*, *RtF3'H*, and *RtMYBF1* increased as the NaCl concentration increased from 100 mM to 300 mM and then decreased under 500 mM NaCl. Under UV-B stress, the transcription of *RtC4H*, *RtOMT*, *RtF3'5'H*, *RtMYBF1*, *RtFLS1*, and *RtFLS2* peaked after 12-h UV-B exposure and decreased with longer exposure. The transcriptions of *RtF3'H*, *RtCHS*, and *RtF3H* increased from 12 to 24 h of UV-B exposure and decreased at 36 h.

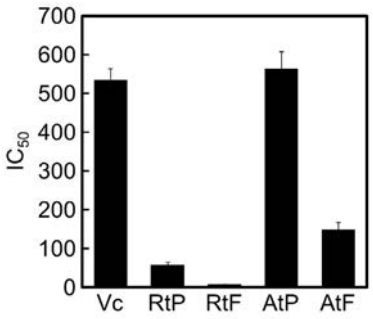

Fig. 3. Antioxidant assay of extracts from *Reaumuria trigyna* and *Arabidopsis thaliana*: Vc - vitamin C (positive control); AtF and AtP - flavonol and polyphenol extracts from *A. thaliana,* RtF and RtP - flavonol and polyphenol extracts from *R. trigyna*.

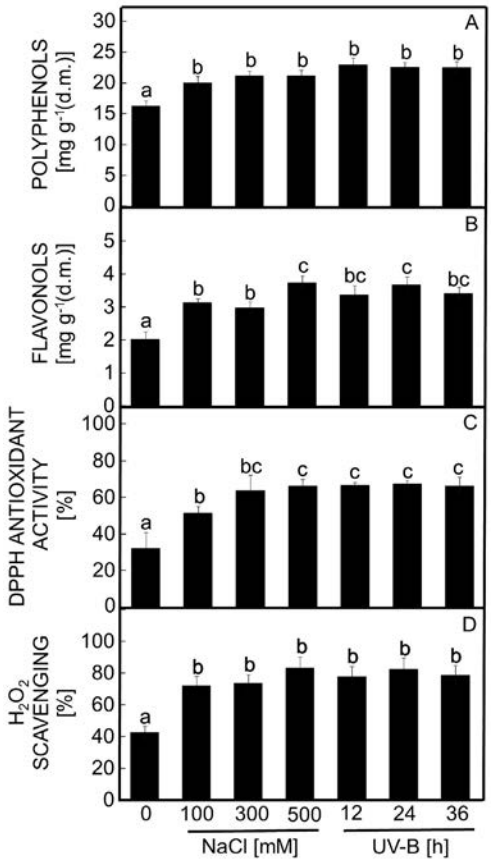

Fig. 4. Measurement of total polyphenol (*A*) and flavonol (*B*) content in *Reaumuria trigyna* under NaCl stress and ultraviolet-B (UV-B) stress. Antioxidant activity of flavonol extracts from *R. trigyna* under NaCl stress and UV-B stress in DPPH assay (*C*). Antioxidant activity of flavonol extracts from *R. trigyna* under NaCl stress and UV-B stress in H_2O_2-scavenging assay (*D*). Means ± SDs (n = 3). Different letters indicate significant differences from control ($P < 0.05$).

The antioxidant potentials of polyphenol and flavonol extracts were compared between *Arabidopsis thaliana* and *R. trigyna* with DPPH as the model free radical. The antioxidant activity is expressed as IC_{50} values (Fig. 3). The flavonol and polyphenol extracts of *R. trigyna* showed lower IC_{50} values than those of extracts from *Arabidopsis thaliana.* The flavonol extracts of *R. trigyna* had the highest antioxidant activity (IC_{50}=8). The polyphenol and flavonol content increased in the seedlings of *R. trigyna* in response to NaCl and UV-B treatments (Fig. 4*A*,*B*). The antioxidant potential of flavonol extracts from *R. trigyna* subjected to NaCl and UV-B treatments was determined by measuring their ability to scavenge H_2O_2 and DPPH. The ability of *R. trigyna* flavonol extracts to scavenge H_2O_2 and DPPH was similar, and the antioxidant activity of flavonol extracts from *R. trigyna* plants subjected to NaCl and UV-B treatments was approximately two-fold that of the control *R. trigyna* plants (Fig. 4*C*,*D*).

We quantitatively analyzed nine flavonols by mass spectrometry to determine changes in their concentrations under NaCl and UV-B treatments. The content of four flavonol components (rutin, isorhamnetin-3-*O*-neohespeidoside, hyperoside, and myricetin) significantly increased ($P < 0.05$) under NaCl and/or UV-B stress (Fig. 5), whereas the content of the other five compounds was unchanged (data not shown). Among the four flavonols, rutin and hyperoside showed dramatically increased content under both UV-B and NaCl stresses. The highest rutin content was at the 300 mM NaCl treatment [0.81 ± 0.036 mg g^{-1}(d.m.)], higher than the hyperoside content [0.023 ± 0.003 mg g^{-1}(d.m.)]. The isorhamnetin-3-*O*-neohespeidoside content was higher under NaCl stress than under UV-B stress. The maximum isorhamnetin-3-*O*-neohespeidoside content was in the 100 mM NaCl treatment [0.043 ± 0.005 mg g^{-1}(d.m.)]. The highest myricetin content was in the 24-h UV-B treatment [0.032 ± 0.005 mg g^{-1}(d.m.)].

Discussion

Many studies have focused on the molecular mechanism of flavonoid metabolism, with evidence of increased expression of several biosynthetic genes, including *PAL*, *F3H*, *CHS*, *FLS*, and *DFR* when plants are subjected to abiotic stresses (Fan *et al.* 2014, Ma *et al.* 2014). In this study on the highly halophytic shrub *R. trigyna*, the transcriptions of genes in the flavonoid biosynthesis pathway were significantly increased under NaCl treatment. The transcript of 57.63 % (68/118) of the unigenes in flavonoid biosynthesis pathways differed significantly between NaCl-treated and control plants. Approximately 70 % of the unigenes in flavonol synthesis pathways were upregulated under NaCl treatment. Genes in flavonol biosynthesis pathways can be upregulated by abiotic stresses, leading to increased flavonol accumulation. For example, drought stress increases the transcription of *CHS*, *F3H*, and *FLS* and flavonol accumulation in *V. vinifera* (Martínez-Lüscher *et al.* 2014). In *Fagopyrum tataricum*, *FtFLS* was upregulated by salicylic acid and NaCl stress, and higher transcription of *FtFLS* was correlated with higher flavonol content (Li *et al*. 2013). In *R. trigyna*, the transcription of *RtFLS1*, *RtFLS2*, *RtCHS*, and *RtF3H3* in the flavonol pathway increased under NaCl and UV-B stresses, and the total flavonol content also markedly increased under these stresses. These results indicated that both NaCl and UV-B treatments induced the expression of genes in the flavonol pathway, leading to increased flavonol content in *R. trigyna.*

Some transcription factors in the MYB family control the expression of genes encoding flavonol pathway enzymes. In *Pinus taeda*, PtMYB1 has been hypothesized to activate transcription by binding to the *PAL* promoter (Bomal *et al*. 2008). In another study, MYBPA1 and MYB5a/b appeared to regulate the expression of *CHI* and *F3'5'H*, both of which are involved in the flavonol

pathway (Matus *et al*. 2009). MYBF1 was shown to specifically activate *FLS* transcription, leading to flavonol production (Czemmel *et al*. 2009). A low expression of *MYBF1* led to rapid decrease in *FLS1* transcription and flavonol content (Czemmel *et al*. 2012). In *R. trigyna*, the expression of *RtMYBF1*, *RtMYB5b* and *RtMYB1* increased under salt stress, together with the expression of flavonol structural genes and the activity of the flavonol biosynthetic pathway. These MYB TFs may regulate the flavonol pathway of *R. trigyna* in response to salt stress.

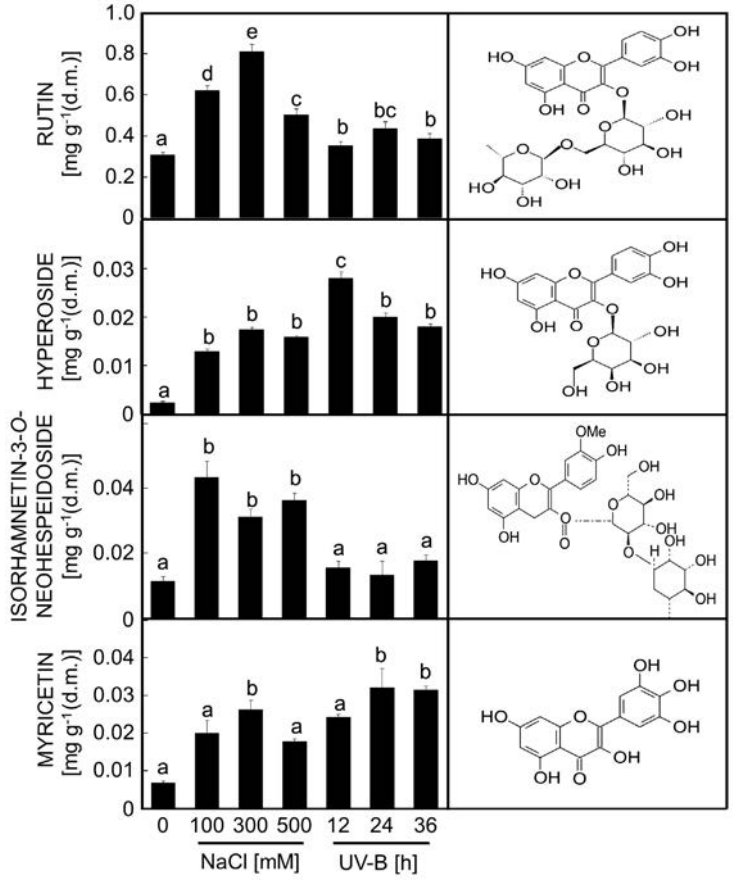

Fig. 5. Contents of four flavonol components (rutin, isorhamnetin-3-O-neohespeidoside, hyperoside, and myricetin) under NaCl stress or UV-B stress. Means ± SDs (n = 3). Different letters indicate significant differences from control ($P < 0.05$).

There are at least 35 flavonol compounds in the model plant *A. thaliana.* The huge chemical diversity of flavonols is because of extensive modifications by enzymes (Saito *et al.* 2013). F3′H and F3′5′H, which catalyze the synthesis of quercetin and myricetin, respectively, are required to produce certain flavonol subclasses (Liu *et al.* 2014). In *R. trigyna,* the expression of *RtF3'H* and *RtF3'5'H* increased under salt and UV-B stresses, as did the myricetin content. Although the quercetin content did not change significantly, the content of quercetin glycosides (rutin, hyperoside and isorhamnetin-3-O-neohespeidoside) increased in response to NaCl and UV-B treatments in *R. trigyna*. UFGT catalyzes the glycosylation of flavonol skeletons, and OMT methylates quercetin to form isorhamnetin (Muzac *et al*. 2000, Jones *et al.* 2003). In *R. trigyna*, the expression of three *RtUFGT* genes and six *RtOMT* genes increased dramatically under salt stress. These genes encode products that may be responsible for the glycosylation of the flavonols that accumulated under the stress treatments. Flavonol glycosides are very strong free radical scavengers that are involved in the responses to many abiotic stresses (Hirano *et al.* 2001). Cold, desiccation, and UV-B can affect the rutin content and activity in *Fagopyrum tataricum* leaves (Suzuki *et al.* 2005). In another study, content of kaempferol and quercetin glycosides increase substantially in *Glycine max* and *Catharanthus roseus* treated with UV-B (Middleton and Teramura 1993, Ferreres *et al.* 2011). Thus, flavonol glycosides are key flavonoid compounds that protect *R. trigyna* plants from salt and UV-B stresses.

Plants growing at high altitudes must have mechanisms to prevent damage caused by high UV-B exposure. For example, maize lines growing at high altitudes develop mechanisms to prevent UV-B damage, such as the accumulation of flavonoids in leaves and silks. These compounds increase the antioxidant capacity of the plant and protect them from UV stress (Casati *et al*. 2005). *R. trigyna* grows in salinized desert regions where there is extraordinarily high UV-B radiation. Our results showed that the antioxidant activity of *R. trigyna* flavonol extracts

was much higher than that of *Arabidopsis* flavonol extracts. Some plants accumulate more flavonoids when they are grown under stress conditions (Agati *et al.* 2012). In this work, *R. trigyna* plants showed increased amounts of flavonol transcripts, increased flavonol content, and increased antioxidant potential when they were grown under UV-B and NaCl stresses. The results showed that the expression of certain genes related to flavonol biosynthesis and the content of some flavonols increased under UV-B and NaCl stresses in *R. trigyna,* leading to increased antioxidant activity, which improved the tolerance of *R. trigyna* to these stresses. Although the expression of *RtC4H, RtFLS2,* and *RtMYBF1* (which are related to flavonol biosynthesis) in *R. trigyna* showed some differences between the NaCl and UV-B treatments, the synthesis of flavonols is tightly regulated by many structural genes and transcription factors (Winkel-Shirley 2001). Thus, the total flavonol content and antioxidant activity did not differ markedly between the two stress treatments; that is, flavonol biosynthesis was similar in the NaCl-treated and UV-B-treated *R. trigyna* plants.

Some gene transcripts and compounds showed non-linear dose-response relationships with the NaCl or UV-B stresses. The causes of such non-monotonic responses remain unclear (Cvrčková *et al*. 2015.). However, it is likely that feedback promotion/inhibition, transcription factors and the factors that regulate them, as well as post-transcriptional regulation mechanisms contribute to the non-linear response curves of certain genes and compounds. In general, our results showed that both genes related to flavonol biosynthesis and the accumulation of certain flavonols was induced by UV-B and salt stress in *R. trigyna*. The results may be caused by these two stresses regulating the response elements of various inducible genes in flavonol biosynthetic pathway. More research is required to explore these ideas. The findings reported in the current study provide new information about the relationships between environmental factors and flavonoid accumulation in *R. trigyna*.

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