Identification of UV-B radiation responsive microRNAs and their target genes in chrysanthemum (Chrysanthemum morifolium Ramat) using high-throughput sequencing

Yanjun Yang1,2, Jiena Guo1,2, Jianmei Cheng1,2, Zhifang Jiang1,2, Ning Xu3,4, Xinyan An1,2, Zehao Chen9, Juan Hao1,2, Siyu Yang5, Zirui Xu5, Chenjia Shen1,2,*, Maojun Xu1,2,*

1 Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, Hangzhou Normal University, Hangzhou, China
2 Key Laboratory of Hangzhou City for Quality and Safety of Agricultural Products, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China
3 Institute of Microbiology, Chinese Academy of Sciences, Beijing, China
4 Beijing Key Laboratory of Detection and Control of Spoilage Microorganisms and Pesticide Residues in Agricultural Products, Beijing University of Agriculture, Beijing 102206, China
5 College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China

A B S T R A C T

Keywords: UV-B radiation bioactive ingredient chlorogenic acid flavonoid glycosylation

MicroRNAs (miRNA) play a key roles in ultraviolet-B (UV-B) induced accumulation of bioactive ingredients in plants. Chrysanthemum (Chrysanthemum morifolium Ramat) is a popular ornamental herb with numerous biological activities, but its miRNAs and their roles in UV-B-induced active ingredient accumulation remain unexplored. High-throughput miRNA sequencing technology was used to identify miRNAs responsive to UV-B radiation. Four libraries were constructed from Chrysanthemum Xiaoyangju leaves harvested at different time points under UV-B radiation. In total, 245 miRNAs belonging to 42 classical families were identified. Under UV-B treatment, 137 miRNAs showed differential expression, including 71 known and 67 novel miRNAs. In addition, 1,779 unique target genes involving diverse biological processes were predicted for 80 known and 85 novel miRNAs. Three key glycolysis genes, Glyceraldehyde-3-Phosphate Dehydrogenase, Pyruvate Kinase, and Glucan Endo-1,3-beta-D-Glucosidase, were predicted as potential target genes of miR396f-5p, PC-5p-294053_21 and miR397a, respectively. Among identified target genes, five key genes involved in the biosynthesis of chlorogenic acids and flavonoids were predicted as potential targets genes of MIR4367-p5, PC-3p-40855_284, MIR393b-p5, and PC-3p-114893_93. The expression of identified miRNAs relating to bioactive ingredient biosynthesis was significantly affected by UV-B radiation, suggesting a strong effect of UV-B radiation on bioactive ingredient accumulation in Chrysanthemum. Interestingly, several miRNAs related to flavonoids biosynthesis such as PC-3p-114893_93 and MIR4367-p5, were inhibited by short-term UV-B radiation, suggesting that the contribution of UV-B to flavonoids accumulation depends on a low dose of UV-B radiation.

1. Introduction

Chrysanthemum (Chrysanthemum morifolium Ramat) has a lengthy history as a traditional folk medicine and is also currently used as an additive in the beverage industry of China (Du et al., 2015). Due to their bioactive ingredients, C. morifolium flowers are also used in daily beverages, especially as a tea. A growing number of studies have shown that the extracts of C. morifolium flowers have several biological activities, including anti-inflammatory, anti-bacterial, anti-oxidant, anti-tumor, and neuroprotective activities (Gao et al., 2016; Jiang et al., 2005; Ukiya et al., 2002). For example, polysaccharides from C. morifolium relieve colitis in rats by regulating the NF-κB/TLR4 and IL-6/JAK2/STAT3 signaling pathways and the intestinal microbiota community (Tao et al., 2018), In recent years, the unique flavor and health benefits of C. morifolium have created increased market demand and expanded its cultivation (Han et al., 2014).

The extracts of C. morifolium flowers have a massive amount of active ingredients, such as chlorogenic acids, flavonoids, glycosides and polysaccharides (He et al., 2018). Flavonoids, a typical class of antioxidants, are primarily responsible for the bioactivities of medicinal C.
morifolium (Yue et al., 2018). The effects of flavonoids extracted from C. morifolium have been evaluated in vitro on MKN45 human gastric cancer cells in vitro have been studied (Liu et al., 2018). A newly-isolated p-hydroxyphenylacetyl flavonoid has also demonstrated cytotoxic activity on human colon cancer cells (Xie et al., 2009). Caffeoylquinic acid, an active phenolic compound enriched in C. morifolium, is considered to be another important pharmacological compound, having antioxidant, anti-HIV and free-radical scavenging properties (Yang et al., 2017). An improved understanding of the metabolic regulation mechanism of these and other compounds in C. morifolium will facilitate effective application of these bioactive substances.

MicroRNAs (miRNAs) are small non-coding RNAs that have been widely identified in plants (Mallory and Vaucheret, 2006), and play diverse biological functions, such as in growth regulation, stress responses, cross breeding, and nutrient absorption (Vidal et al., 2013). Recent studies have also shown that miRNAs played important roles in the metabolic process of plants. In tomato (Solanum tuberosum L.), several light-responsive miRNAs were reported to be involved in secondary metabolisms, including alkaloids metabolism, uridine monophosphate (UMP) salvage, lipid metabolism, and cellulose catabolism (Qiao et al., 2017). Light-induced expression of miR858a plays a potential role in flavonoid biosynthesis by regulating the phenylpropanoid pathway (Sharma et al., 2016). Meanwhile, drought-induced expression of miRNA156 is involved in the induction of plant anthocyanin biosynthesis (Gonzalez-Villagra et al., 2017).

Recently, the identification of C. morifolium microRNAs has attracted considerable attention. In 2015, a microRNA expression profiling study identified that miR159a, miR160a and miR393a may be involved in the responses to aphid infestation (Xia et al., 2015). Another comparative profiling study revealed 69 chrysanthemum miRNAs that have been differentially expressed between normal and abnormal embryos of C. morifolium (Zhang et al., 2015b). Additionally, 16 C. morifolium miRNA families and their 13 CmDOF target genes of have been identified under phytohormone treatments and abiotic stresses (Song et al., 2016). However, much about the roles of miRNAs in C. morifolium responses to stress remains to be elucidated.

Ultraviolet-B (UV-B) radiation is a component of solar radiation, and high-dose UV-B exposure harms damage to the growth and development of plants (Gill et al., 2015; McKenzie et al., 2007). Conversely, low-dose UV-B exposure increases secondary metabolite accumulation in various plants (Gu et al., 2010); in industrial production, UV-B has been frequently used as a productive elicitor to increase the accumulation of active metabolites (Pandey and Pandey-Rai, 2014). For example, catharanthine in Catharanthus plants, glycyrrhizin in Glycyrrhiza plants and taxol in Taxus trees are all significantly induced by UV-B treatment (Afreen et al., 2005; Ramani and Chelliah, 2007; Zu et al., 2010). Previous studies have investigated the effects of UV-B radiation on the accumulation of bioactive compounds accumulation in the flowers of multiple C. morifolium species (Yao et al., 2014), in which several levels of UV-B treatments were found to affect biochemical reactions and promote the accumulation of postharvest bioactive ingredients in flowers of C. morifolium (Si et al., 2015; Yao et al., 2015). However, the miRNA-level responses of the genus C. morifolium to UV-B radiation are currently largely unknown.

Using miRNA sequencing, followed by bioactive ingredient determination, we identified miRNAs responsive to low-dose UV-B radiation and their expression patterns. Our results provide clues for identifying candidate miRNAs involved in UV-B-induced active ingredient accumulation.

2. Materials and Methods

2.1. UV-B treatment and plant material sampling

The C. morifolium cultivar ‘Xiaoyangju’ was used in our study. All seedlings were produced from tissue culture technology and planted in a greenhouse at Hangzhou Normal University (Hangzhou, China) with an average temperature of 26 °C, a light/dark cycle of 16/8 h, and a relative humidity of 65%. Two-month-old plants were randomly classed into four groups, with three replicates each. Three groups were exposed to 10 μmol m⁻² s⁻¹ UV-B radiation for 3 h, 6 h, and 12 h, respectively. One group of plants without UV-B radiation exposure served as the
control. The UV-B radiation was artificially generated by a UV fluorescent lamp with Q-Lab narrowband UV-B tubes, and UV-C radiation was filtered out by means of 3-mm transmission cutoff filters (Schott, Mainz, Germany) (Fig. 1a). Fresh leaves were harvested from all seedlings groups and stored in liquid N2 until further use.

2.2. RNA isolation and small RNA (sRNA) library construction

Total RNA was isolated with TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer’s protocol. RNase-Free DNase I (Promega, Madison, USA) was added to remove DNA contamination. Equal amounts of RNA from the control and the three UV-B treated groups were used for sRNA library construction.

Construction of sRNA libraries was performed by LC-Bio (Hangzhou, China). Briefly, sRNA fragments with lengths ranging 18 to 30 nt were isolated using a 15% denaturing polyacrylamide gel. Then, the 3′ and 5′ RNA sequencing adapters were ligated to the RNA fragments using RNase-Free DNase I and T4 RNA ligase, and cDNA constructs were created using reverse transcription followed by PCR. Finally, the purified PCR products were sequenced on a HiSeq 2500 system (Illumina, San Diego, USA) according to the manufacturer’s protocol.

2.3. Bioinformatic analysis

After sequencing, low-quality reads were filtered out to generate clean reads. The clean reads were BLASTed against the NCBI GenBank, Repeat and Rfam databases, to identify and exclude known non-coding RNAs. Additionally, protein-coding sequences present in C. morifolium reference transcriptome (Yang et al., 2018) were discarded. The remaining sRNA sequences were aligned with known miRNA sequences from miRBase 19.0, allowing a maximum of two mismatches, and miRNA stem-loop structure were predicted using RNAfold software.

Novel and known miRNAs were screened basing on standard criteria (Meyers et al., 2008). Briefly, unique sequences mapping to the hairpin arms of mature miRNAs in specific species were identified as known miRNAs. Meanwhile, unique sequences mapping to the opposite arm of known specific precursors were tRNAs. The remaining sequences that mapped to the precursors of other selected species in miRBase 22.0 were also defined as known miRNAs. For each identified miRNA, the read count, length and nucleotide bias at each nucleotide position were determined.

2.4. Analysis of differentially expressed miRNAs

To evaluate the expression abundance of miRNAs, read counts were normalized according to the transcripts per million method. Differential expression analysis of two sample groups was carried out using the R package DESeq. The threshold of statistical significance between groups was defined as \( P < 0.01 \) and \( \log_{2}(\text{fold change}) > 1 \). Differentially expressed miRNAs were clustered using MeV software with the K-means method.

2.5. Target gene prediction and enrichment analysis

In our study, putative miRNA target genes were predicted from the C. morifolium transcriptome (Yang et al., 2018) based on sequence similarity using PsRobot (Wu et al., 2012). Enrichment analysis of the candidate target genes was performed using Gene Ontology (GO) functional terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG). A two-tailed Fisher’s exact test was applied to identify enriched terms, with corrected P-values < 0.05 considered as significant. The background consisted of all miRNAs in the respective databases.

2.6. Measurement of bioactive ingredients in Chrysanthemum leaves

Total soluble sugar contents was determined as described previously (Yu et al., 2016). Briefly, C. morifolium leaves were weighed and then extracted in 80% ethanol at 80°C for 20 min. The supernatant was filtered, and 1 mL of extract was incubated with 5 mL of ethanone reagent at 95°C for 10 min. Finally, the absorbance was measured at 620 nm. The total flavonoid and chlorogenic acid contents were determined as in our previous work (Yang et al., 2018). Standards of chlorogenic acid, caffeic acid and rutin were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.7. Dual-Luciferase Report Assay

To create effector constructs, the mature sequences of MIR156a and MIR393b-p5 were subcloned into the pCAMBIA 1301 vector. For reporter constructs, the MIR156a-binding regions of Squamosa Promoter Binding Protein-like 9 (SPL9) and the MIR393b-p5-binding regions of Phenylalanine Ammonia Lyase (PAL) were subcloned into the pGreenII 0800-Luc vector. Then, the effector and reporter constructs were co-transformed into tobacco leaves and leaf samples were collected. The same leaf samples were used for both plant tissue extraction and dual-luciferase assay. Leaf discs (around 20 mg) were ground to a fine powder in liquid nitrogen and homogenized in Passive Lysis Buffer (Promega, Madison, WI, USA). The extract was clarified of non-dissolved plant tissues by performing a quick spin at 12,000 g, 4°C, for 60 s. Twenty microliters of supernatant were used for dual-luciferase assay with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) on a 96 Microplate Luminometer equipped with dual injectors.

2.8. Validation of differentially expressed miRNAs

Stem-loop quantitative reverse transcription PCR was carried out to determine the expression of the selected miRNAs. The specific stem-loop reverse transcription primers, the forward primers for miRNAs, and the universal reverse primer were designed following the method described by Chen et al. (2005). Total mRNA and miRNA were extracted from the C. morifolium leaves using a miRNA Purification Kit (CoWin Biotech, Beijing, China) and a RNApure Plant Kit (CoWin Biotech, Beijing, China), respectively. RNAs were reverse transcribed into cDNA using a HiFIScript cDNA Synthesis Kit (CoWin Biotech, Beijing, China). The PCR amplification conditions were as follows: 50°C for 3 min; 95°C for 2 min; 39 cycles at 95°C for 15 s and 60°C for 30 s; 4°C overnight. Relative fold changes were calculated based on the comparative cycle threshold (2-\( \Delta\Delta C_{T} \)) values. For qPCR analysis of miRNAs, U6 was used as an internal reference.

2.9. Statistical analysis

Significant differences between two sample groups were determined by a one-way analysis of variance with a Student’s t-test at a significance level lower than 0.05. Analyses of gene expression as well as compound content were performed based on three biological replicates.

3. Results

3.1. Effects of UV-B radiation on bioactive ingredient accumulation in C. morifolium

Our study determined the soluble sugar, total flavonoid, neochlorogenic acid and chlorogenic acid contents in C. morifolium leaves under UV-B radiation. Control leaves were found to contain 32.4 mg.g\(^{-1}\) of soluble sugar, which was significantly up-regulated by UV-B exposure to 36.9 mg.g\(^{-1}\) and 41.8 mg.g\(^{-1}\) at 3 h and 12 h, respectively (Fig. 1b). The total flavonoid content of control leaves was 201.1 mg.g\(^{-1}\), which with UV exposure was raised to 265.4 mg.g\(^{-1}\) and 257.3 mg.g\(^{-1}\) at 6 h and 12 h, respectively (Fig. 1c). The neochlorogenic acid content of the control leaves was 0.074 mg.g\(^{-1}\), which was increased to...
0.11 mg·g⁻¹ and 0.186 mg·g⁻¹ at 6 h and 12 h, respectively (Fig. 1d). The chlorogenic acid content of the controls was 14.3 mg·g⁻¹, and was raised to 21.1 mg·g⁻¹ and 32.3 mg·g⁻¹ at 6 h and 12 h, respectively (Fig. 1e).

3.2. Overview of C. morifolium microRNAome

In our study, four libraries of small RNAs were established and sequenced from C. morifolium leaves given different UV-B exposure treatments. After filtering, 147,266,889 valid reads were obtained, 37.2% of which were unique reads (Table S1). Length distribution analysis showed that the majority of reads from each sample were 24 bp in length (Fig. 2a). Noncoding RNAs other than miRNAs, including rRNA, tRNA, snoRNA, and snRNA, were filtered out; most non-miRNAs classified as rRNA or tRNA (Fig. 2b). In total, 340 distinct miRNAs were identified in at least one sample group, with 170 miRNAs being identified in all four sample groups (Table S2). A Venn diagram revealed that there were five miRNAs specific to the control group, 15 for the UV-B 3 h group, three for the UV-B 6 h group, and 15 for the UV-B 12 h group (Fig. 2c).

3.3. Identification of known and novel miRNAs

All of the miRNAs identified were classified into five groups based on their mapping features: 1, 2a, 2b, 3, and 4. Groups 1-3 consisted of known miRNAs, which mapped to the selected miRNAs/pre-miRNAs in miRBase (Fig. 2d). Group 4 consisted of miRNAs that could not be mapped to the selected pre-miRNAs (novel miRNAs), and contained the majority of identified miRNAs.

3.4. Classification and conservation analysis of miRNA families

All identified miRNAs were additionally classified into 42 classical families. The largest families were MIR168 and MIR156, consisting of ten miRNAs each. Another seven families (MIR398, MIR393, MIR396, MIR169_2, MIR171_1, MIR169_1, and MIR166) contained more than five miRNAs (Fig. 3a). Most C. morifolium miRNAs displayed significant similarities to several known miRNAs in other plant species. For example, 165 miRNAs showed significant similarities to known miRNAs in soybean (Glycine max), 147 miRNAs showed significant similarities to known miRNAs in apple (Malus domestica), and 134 miRNAs showed significant similarities to known miRNAs in poplar (Populus trichocarpa) (Fig. 3d). Total miRNA nucleotide bias and first nucleotide bias are additionally shown in Fig. 3b and c.

3.5. Identification of UV-B responsive miRNAs of C. morifolium

A number of differentially expressed miRNAs (DEMs) were identified in the UV-B treatments. An overview of transcript expression variation was produced using PCA, showing four separate sample groups (Fig. S1). In total, 137 miRNAs, including 71 known and 67 novel miRNAs, showed differential expression under UV-B treatment (Fig. 4a and Table S3). The DEMs grouped into nine clusters, I to IX, which contained the UV-B responsive miRNAs identified for each time point. Cluster IX miRNAs were responsive to UV-B treatment at 3 h; Cluster VIII miRNAs were responsive to UV-B treatment at 6 h; and Cluster V miRNAs were responsive to UV-B treatment at time 12 h. Interestingly, the miRNAs belonging to Cluster III were down-regulated and those in Clusters I, V and IX were up-regulated by UV-B treatment at all three time points (Fig. 4b). The numbers of up- and down-regulated miRNAs in each comparison are given in Fig. 4c.

3.6. Target prediction of the DEMs

3.6.1. Prediction of DEM targets

To further analyze the biological functions of C. morifolium miRNAs, putative targets were predicted for the identified DEMs using the
A total of 1,779 unique target genes were predicted for 80 known and 85 novel miRNAs, with each miRNA having multiple predicted target genes involving diverse biological processes (Table S4). Most of the predicted target genes were grouped into three major GO categories. Within the biological process category, the most abundantly represented GO terms were “regulation of transcription,” “transcription,” and “protein phosphorylation.” Within the cellular component category, the most abundant terms were “nucleus,” “plasma membrane,” and “plasmodesma.” Within the molecular function category, the most abundant terms were “protein serine/threonine kinase activity,” “ATP binding,” and “DNA binding” (Fig. S2).

In addition, all predicted target genes were evaluated for enrichment across the 107 KEGG pathways (Table S5). The most significantly enriched KEGG pathways were “plant-pathogen interaction” (ko04626), “amino sugar and nucleotide sugar metabolism” (ko00520), “mRNA surveillance pathway” (ko03015), “pantothenate and CoA biosynthesis” (ko00770), and “ABC transporters” (ko02010) (Fig. 5).

3.7. Identification of glycolysis-related miRNAs and their target genes

The predicted targets of DEMs included three key glycolysis genes: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Pyruvate Kinase (PK), and Glucan Endo-1,3-beta-D-Glucosidase (GEG) (Fig. 6a). Specifically, GAPDH was a potential target of csi-miR396f-5p, PK was a potential target of PC-5p-294053_21, and GEG was a potential target of ath-miR397a (Fig. 6a). Furthermore, expression of miR396f-5p was significantly down-regulated after 12 h of UV-B treatment, while the expression of PC5p-294053_21 was significantly up-regulated. Interestingly, the expression of miR397a was significantly up-regulated at all three time points (Fig. 6b).

3.8. Identification of chlorogenic acid- and flavonoid-related miRNAs and their target genes

The predicted DEM targets included five key genes involved in the biosynthesis of chlorogenic acid and flavonoids: Anthocyanidin Synthase (ANS), Flavanone 3-Hydroxylase (F3H), 4-Coumarate:CoA Ligase (4CL), PAL, SPL9 and UDP-Glucuronosyltransferase (UGT88). Specifically, the genes ANS and F3H were potential targets of MIR4367-p5, 4CL was a potential target of PC-3p-294053_21, PAL was a potential target of MIR393b-p5, the SPL9 gene was a potential target of ptc-miR156a, and UGT88 was a potential target of PC-3p-114893_93 (Fig. 7a). Four of these miRNAs (miR4367-p5, PC-3p-114983_93, ptc-miR156a and miR393b-p5) were significantly up-regulated after 12 h of UV-B
treatment. Interestingly, the expression of PC-3p-40855_284 was significantly up-regulated at all three time points (Fig. 7b).

3.9. SPL9 and PAL are target genes of MIR156a and MIR393b-p5, respectively

Dual-luciferase reporter gene assays were used to verify the predicted interaction between selected microRNAs and their putative target genes. Dual-luciferase reporter vectors were constructed with either wild type (WT) or mutated (Mut) fragments of the miRNA-binding regions of SPL9 for MIR156a and PAL for MIR393b-p5. The results showed that MIR156a and MIR393b-p5 suppressed luciferase activity in WT-transfected cells, but not in Mut-transfected cells (Fig. 8). These results validate that SPL9 is a target of MIR156a and PAL is a target of MIR393b-p5.

3.10. qRT-PCR analysis of miRNAs related to bioactive ingredient biosynthesis

The expression patterns of seven key miRNAs related to bioactive ingredient biosynthesis were confirmed using qRT-PCR. The expression levels obtained from this assay were basically consistent with the RNA-seq results (Fig. 9). Most of the selected miRNAs were significantly up-regulated after 12 h of UV-B exposure, except for miR396f-5. MiR397a was up-regulated at all three time points, and expression levels of PC-3p-114893_93 and MiR4367-p5 were first reduced at 6 h and then elevated at 12 h of UV-B exposure.

4. Discussion

*C. morifolium* is a well-known floricultural and medicinal crop that has been cultivated throughout the world for many years (Shinoyama et al., 2012). Tea made from *C. morifolium* flowers, which contains various bioactive ingredients, is considered to be beneficial to human health (Li et al., 2019). The production of these bioactive ingredients is at least partially regulated by miRNAs, whose expression is in turn affected by environmental conditions. Our study identified a number of *C. morifolium* miRNAs responsive to UV-B radiation.

A large number of highly-conserved miRNAs in *C. morifolium* plants have been identified as having functions in growth, development, differentiation, metabolic regulation, and responses to environmental stimulus (Dong et al., 2016). In *C. nankingense*, 81 root-specific miRNAs and 101 leaf-specific miRNAs were identified as being nitrogen starvation-responsive (Song et al., 2015). In another study, 228 chrysanthemum miRNAs and 1,037 miRNA target genes had significant changes in expression frequency during embryonic development.
Furthermore, 303 conserved miRNAs and 234 potential novel miRNAs have been detected in *C. morifolium* leaves (Xia et al., 2015). Recently, a total of 170 miRNAs were identified as being expressed in the embryo after pollination, including miR169b, miR440, miR528-5p, miR164c and miR159a (Zhang et al., 2017).

The identification of UV-B responsive miRNAs in plants has been a hot topic, receiving considerable attention in recent years. For example, 21 microRNA genes in 11 microRNA families are known to be up-regulated by UV-B treatment in the model plant *Arabidopsis* (Zhou et al., 2007). In *Populus tremula*, 24 UV-B responsive miRNAs were identified, including 13 up- and 11 down-regulated miRNAs (Jia et al., 2009). In wheat, six miRNAs were highly responsive to UV-B treatment, and the promoters of UV-B responsive miRNAs contained some light-relevant cis-elements, such as the I-box and G-box (Wang et al., 2013). Recently, a role has been revealed for UV-B light in regulating sRNAs during grapevine berry development, and several grape miRNAs to be up-regulated by UV-B treatment, such as miR156, miR482, miR530, and miR828 (Sunitha et al., 2019).

Our study identified 245 miRNAs in total, which is similar to the reports of Xia (2015) and Zhang (2017), and greater than the numbers from Song (2015) and Zhang (2017). The large total number of identified miRNAs gave us an opportunity to explore the potential involvement of novel *C. morifolium* miRNAs in UV-B-induced bioactive ingredient accumulation. We identified 137 UV-B responsive miRNAs, which is more than have been identified in other plants. This large number of UV-B responsive miRNAs suggests a considerable effect of UV-B radiation on transcriptional regulation in *C. morifolium*.

The glycolysis pathway converts glucose into pyruvate and provides intermediates and energy for plant growth and development (Beurton-Aimar et al., 2011). Several glycolysis-related genes have been identified as potential targets of miRNAs in plants (Lukasik et al., 2013). For example, brmiR168 in cabbage (*Brassica rapa*) targets the glycolysis gene Pyruvate Kinase (Dhandapani et al., 2011). In camellia fruit (*Camellia oleifera*), miR156 plays an important role in the regulation of glycolysis and nutrient transformation genes (Liu et al., 2019). Glycolysis-related genes in chrysanthemum have been identified based on homology to model plants (Yang et al., 2018). Among these, our study identified three key genes as potential targets of miRNAs, indicating an involvement of miRNAs in the regulation of *C. morifolium* glycolysis.

PK is a conserved enzyme that catalyzes the reversible conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate (Banks et al., 1979). *C. morifolium* PK was predicted as a target of PC-5p-294053_21, a novel
miRNA that was significantly up-regulated after 12 h of UV-B treatment, suggesting that UV-B-induced PC-5p-294053_21 might play a role in the balance of glycerate-1, 3-P2, and pyruvate. Additionally, we identified the gene encoding endo-1,3-β-glucanase as a target of miR397a, which was up-regulated by UV-B treatment. Endo-1,3-β-glucanases are reported to play roles in the synthesis of polysaccharides (Takeda et al., 2015); therefore, our data suggests that miR397a is involved in the UV-B-induced accumulation of polysaccharides in C. morifolium.

The phenylpropanoid pathway is a gateway for the production of many secondary metabolites, such as flavonoids and chlorogenic acids (Dehghan et al., 2014; Tohge and Fernie, 2017). In plants, core units for basic phenylpropanoid metabolism are provided by several intermediates of the shikimate pathway (Vogt, 2010). Previous studies have investigated effects on phenylpropanoid metabolism-related genes from various environmental stresses, such as UV-B radiation. For example, short-term UV-B radiation increases anthocyanin levels in plants (Debski et al., 2016). The penultimate step in the biosynthesis of the anthocyanin class of flavonoids is catalyzed by Anthocyanidin Synthase (ANS), and the encoding gene ANS was found to be up-regulated by UV-B in plants (Xu et al., 2008). In C. morifolium, ANS was identified as a target of MIR4367-p5, expression of which was significantly reduced at 3 h and 6 h of UV-B radiation and elevated at 12 h. These results suggest that short-term UV-B radiation might inhibit MIR4367-p5, which allows greater expression of ANS. Additionally, PC-3p-114893_93 was significantly down-regulated at 6 h and up-regulated at 12 h. Among its targets was UGT88, another important gene in anthocyanin biosynthesis (Nakatsuka and Nishihara, 2010). Taken together, our data indicates that anthocyanin accumulation in C. morifolium depends on a low dose of UV-B radiation.

**Fig. 6. Identification of glycolysis-related miRNAs and their target genes.** (a) Overview of glycolysis in Chrysanthemum. Target genes were identified for csi-miR396f-5p, PC-5p-294053_21 and ath-miR397a. Enzyme abbreviations are: GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PK: pyruvate kinase; and GEG: glucan endo-1,3-beta-D-glucosidase. (b) Expression changes of miRNAs associated with glycolysis under UV-B treatment in Chrysanthemum. **"** indicates significant change (P < 0.05) in expression level between control and UV-B treatment.
5. Conclusion

In conclusion, 137 miRNAs in *C. morifolium*, including 71 known and 67 novel miRNAs, showed differential expression under UV-B radiation. Among the targets of UV-B responsive miRNAs were three glycolysis-related genes (*GAPDH*, *PK*, and *GEG*) and five chlorogenic acid- and flavonoid-related genes (*ANS*, *F3H*, *4CL*, *PAL*, and *UGT88*), suggesting a considerable effect of UV-B radiation on the accumulation of bioactive ingredients in *C. morifolium*.

Funding

This study was funded by the National Natural Science Foundation of China (Grant No. 81803655), the Zhejiang Provincial Natural Science Foundation of China (Grant No. LY18H2800012, LY19C150005, LY19C020003, and LY19C160001), the National Natural Science Foundation of China (Grant No. B1673539, B1373907 and 31601343), the Scientific Research Fund of Zhejiang Province Education Department (Grant No. Y201533081), Major Increase Or Decrease Program in The Central Finance Level (Grant No. 2060302), the Zhejiang Provincial Key Research & Development Project Grants (Grant No. 2017C02011, 2018C02030), Hangzhou Normal University Xinmiao Talent Program (2019R426078).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

**Yanjun Yang**: Conceptualization, Data curation, Resources, Funding acquisition, Validation, Writing - original draft. **Jiena Guo**: Data curation, Investigation, Software. **Jianmei Cheng**: Data curation, Investigation, Software, Visualization. **Zhifang Jiang**: Formal analysis. **Ning Xu**: Software, Funding acquisition. **Xinyan An**: Data curation, Resources, Investigation, Methodology. **Zhehao Chen**: Investigation, Methodology, Writing - original draft. **Juan Hao**: Data curation, Resources, Supervision, Visualization. **Siyu Yang**: Data curation,
Investigation. Zirui Xu: Data curation, Investigation. Chenjia Shen: Conceptualization, Writing - review & editing. Maojun Xu: Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112484.

Fig. 8. Validation of predicted miRNA-target interactions by dual-luciferase reporter gene assay. (a) Schematic of wild type (WT) and mutated (MUT) MIR156a-binding regions of SPL9. (b) The interaction between MIR156a and SPL9 was verified by dual-luciferase reporter gene assay. **P < 0.05 as determined by t-test. (c) Schematic of wild type (WT) and mutated (MUT) MIR393b-p5-binding regions of PAL. (d) The interaction between MIR393b-p5 and PAL was verified by dual-luciferase reporter gene assay. ***P < 0.05 as determined by t-test.

Fig. 9. Validation of bioactive ingredient-related miRNAs expression using qRT-PCR. Eight miRNAs, including miR396f-5p, PC-5p-294053_21, miR397a, miR3575-p5, PC-3p-114893_93, PC-3p-40855_284_93, PC-3p-40855_284, MIR393b-p5 and miR156a, were checked by qRT-PCR. Error bars indicate SD of three biological replicates. **P < 0.05 indicates a statistically significant difference.

References


