UV-B Radiation Largely Promoted the Transformation of Primary Metabolites to Phenols in Astragalus mongholicus Seedlings

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Abstract: Ultraviolet-B (UV-B) radiation (280–320 nm) may induce photobiological stress in plants, activate the plant defense system, and induce changes of metabolites. In our previous work, we found that between the two Astragalus varieties prescribed by the Chinese Pharmacopoeia, Astragalus mongholicus has better tolerance to UV-B. Thus, it is necessary to study the metabolic strategy of Astragalus under UV-B radiation further. In the present study, we used untargeted gas chromatography-mass spectrometry (GC-MS) and targeted liquid chromatography-mass spectrometry (LC-MS techniques) to investigate the profiles of primary and secondary metabolic. The profiles revealed the metabolic response of Astragalus to UV-B radiation. We then used real-time polymerase chain reaction (RT-PCR) to obtain the transcription level of relevant genes under UV-B radiation (UV-B supplemented in the field, $\lambda_{\text{max}} = 313$ nm, 30 W, lamp-leaf distance = 60 cm, 40 min·day$^{-1}$), which annotated the responsive mechanism of phenolic metabolism in roots. Our results indicated that supplemental UV-B radiation induced a stronger shift from carbon assimilation to carbon accumulation. The flux through the phenylpropanoids pathway increased due to the mobilization of carbon reserves. The response of metabolism was observed to be significantly tissue-specific upon the UV-B radiation treatment. Among phenolic compounds, C6C1 carbon compounds (phenolic acids in leaves) and C6C3C6 carbon compounds (flavones in leaves and isoflavones in roots) increased at the expense of C6C3 carbon compounds. Verification experiments show that the response of phenolics in roots to UV-B is activated by upregulation of relevant genes rather than phenylalanine. Overall, this study reveals the tissues-specific alteration and mechanism of primary and secondary metabolic strategy in response to UV-B radiation.

Keywords: Astragalus mongholicus; ultraviolet-B radiation; phenolics; untargeted gas chromatography-mass spectrometry; targeted liquid chromatography-mass spectrometry

1. Introduction

Astragalus mongholicus (A. mongholicus) is an important perennial herb of the Legumes family [1,2]. Its dried roots (Radix astragali) are one of the most popular Chinese herbal medicines in East Asia and are considered as healthy food in Western countries [3,4]. Radix astragali is often used as an
antiperspirant, a vimmuno-stimulant, and a supplementary medicine during cancer therapy [5–7].

Calycosin-7-O-β-d-glucoside (CAG) can potentially be used as a “marker compound” for the chemical evaluation or product standardization of Radix astragali [8–10].

The need for A. mongholicus to capture sunlight for photosynthesis inevitably exposes the plant to ultraviolet (UV) radiation. Ultraviolet radiation is the general term for three radiation wavelengths: UV-C (200–280 nm), UV-B (280–320 nm), and UV-A (320–400 nm). Ultraviolet-C is completely absorbed by atmospheric gases. Ultraviolet-A is barely absorbed by atmospheric ozone, but UV-A impact on plants is small. Ultraviolet-B is potentially harmful to plants but is partially absorbed by ozone [11–13]. It was reported that the influx of UV-B radiation will likely increase as a result of the depletion of stratospheric ozone [14]. Increased amounts of UV-B radiation affect plant development, morphology, and physiology [15]. The sessile lifestyle of plants forces them to adapt to dynamic environmental conditions. To counteract these problems, plants use a range of strategies, including increases in leaf thickness, UV-B reflective properties, and the accumulation of UV-B-absorbing secondary metabolites. The most common protective mechanism against potentially damaging irradiation is the biosynthesis of UV-absorbing compounds [16]. These secondary metabolites mostly consist of phenolic compounds, flavonoids, and hydroxycinnamates, which accumulate in the vacuoles of epidermal cells in response to UV-B radiation and attenuate the penetration of the UV-B portion of the solar spectrum into deeper cell layers with little effect on the visible region. These responses to UV-B radiation may result in the reprogramming of metabolites in A. mongholicus and even altered accumulation of bioactive compounds. Given this fact, it is essential to better understand the adaptive responses of metabolites in A. mongholicus to increased UV-B radiation.

Phenolic compounds play diverse roles in plants. These compounds provide structural support of the cell wall or protect plants against pathogens, herbivores, and UV radiation [17]. Of all classes of secondary metabolites, phenolics, specifically flavonoids, are the most relevant for UV protection. Plant phenolics are compounds having at least one aromatic ring substituted with at least one hydroxyl group. The hydroxyl group can be free or engaged in another function as an ether, ester, or glycoside [18–20]. Phenolics exhibit a large variety of structures in nature and can be divided into three groups according to their chemical structure: (1) compounds having a C6C1 carbon skeleton, such as 4-hydroxybenzoic acid, vanillic acid, and salicylic acid; (2) compounds having a C6C3 carbon skeleton, such as caffeic acid, p-coumaric acid, and ferulic acid; and (3) compounds having a C6C3C6 carbon skeleton (flavonoids are typical C6C3C6 phenolic compounds) [21,22]. Phenolic compounds are generally synthesized via the shikimate pathway. The shikimate pathway is a major biosynthetic route for both primary and secondary metabolism. This pathway begins with phosphoenolpyruvate and erythrose-4-phosphate and ends with chorismate [23,24]. Phenylalanine, a key metabolite, is synthesized by chorismate [25]. Phenylalanine is considered the general precursor of C6C1-, C6C3-, and C6C3C6 compounds and their polymers in plant metabolism [26]. Many investigations have closely focused on the accumulation of phenolic compounds, which is regulated by biotic and abiotic stress [27]. Astragalus mongholicus is considered a rich source of natural phenolic compounds [28,29].

Regarding the complex primary and secondary metabolites fluctuations of higher plants, metabolomics plays a vital role as a suitable tool [15,30]. Metabolomics approaches are becoming more widely used in modern biology [28,31,32]. In plant metabolomics studies, common analytical technologies include liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance spectroscopy (NMR), among others [33–35]. Gas chromatography-mass spectrometry is widely used in metabolomic studies due to its high quality and reproducibility, wide dynamic range, universal mass spectral library and ability to detect hydrophilic metabolites after derivatization [36]. A reference pool of GC-MS for many primary metabolites, such as sugars, organic acids, fatty acids, and amino acids, has been established [37–39]. The short run time and relatively low running cost are strong advantages of GC-MS [40]. In contrast, LC-MS can potentially analyze a wide variety of large hydrophobic metabolites predominant in secondary compounds in plants; these compounds include phenolic compounds, terpenoids and...
Moreover, the development of the ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) methods leads to more powerful analyses of metabolites because of its higher throughput and shorter run time than those of conventional HPLC-MS [44,45]. Accordingly, the combination of GC-MS and LC-MS could clearly enhance the coverage of metabolites, allowing both a comprehensive overview and detailed analysis of metabolic changes in plants [46]. Recently, these two methods have been jointly adopted for many botanical studies [30,47–49].

Many of the biosynthetic enzymes of phenolics, such as phenylalanine ammonia lyase (PAL, E.C. 4.3.1.25) and chalcone synthase (CHS, E.C. 2.3.1.74), are activated by UV-B [1,50]. Flavonoid biosynthesis and their regulation have been thoroughly investigated. As the major bioactive compound, the calycosin 7-O-glucoside (CAG) biosynthesis pathway has been completely elucidated based on its presence in other legumes [10,51–53]. Calycosin 7-O-glucoside is synthesized from L-phenylalanine via the isoflavonoid branch of phenylpropanoid metabolism. PAL, cinnamate 4-hydroxylase (C4H, E.C. 1.14.13.11) and 4-coumarate-coenzyme A ligase (4CL, E.C. 6.2.1.12) are enzymes involved in the upstream general phenylalanine pathway. Isoliquiritigenin, an isoflavonoid skeleton, is synthesized via CHS and chalcone reductase (CHR), co-catalyzed by the condensation of 4-coumaroyl-CoA and three molecules of malonyl-CoA. Afterward, a series of chemical reactions is performed under the catalysis of chalcone isomerase (CHI, E.C. 5.5.1.6), flavonone synthase (IFS, E.C. 1.14.13.86), isoflavone O-methyltransferase (IOMT) and isoflavone 3′-hydroxylase (I3′H). At the last step, the formation of CAG from calycosin (CA) is catalyzed by uridine diphosphate glucose (UDP)-glucose: calycosin 7-O-glucosyltransferase (UCGT). For the last step, the formation of CAG from CA is catalyzed by uridine diphosphate glucose (UDP)-glucose: calycosin 7-O-glucosyltransferase (UCGT) [54,55].

Due to the release of anthropogenic pollutants such as chlorofluorocarbons, a larger proportion of the UV-B spectrum reaches the surface of the earth and affects all living organisms [56,57]. In general, the most common protective mechanism against potentially damaging irradiation in plants is the biosynthesis of UV-absorbing compounds, such as phenolic compounds, flavonoids, and hydroxycinnamate esters [16]. The use of UV-B radiation is expected to induce the accumulation of CAG in A. mongholicus [55,58]. This could satisfy consumer demand for these naturally derived health-promoting products. Our goal is to understand the response of the metabolites in A. mongholicus to UV-B radiation. To the best of our knowledge, no information on the metabolites response mechanism of A. mongholicus to UV-B radiation is currently available. To deeply explore the response and mechanism explaining how metabolic reprogramming can achieve a new steady state under increased UV-B radiation, we investigated the influence on the specific tissue accumulation of primary and phenolic metabolites using untargeted (GC-MS) and targeted (LC-MS) metabolomics in A. mongholicus under increased UV-B radiation.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Treatments

Seeds of A. mongholicus were sown in Botanical Garden of the Key Laboratory of Plant Ecology, Northeast Forestry University, Harbin, northeastern of China (natural environment at east longitude 126°38′, north latitude 45°43′). All plants grew under standard field conditions, with an average temperature of 16 °C during the day (14 h) and 4 °C at night (10 h), and water was applied every two days. After three months, plants of uniform height and stem thickness were transferred to containers (37 cm × 28 cm × 10 cm) and used for this study. All containers (n = 6) contained equal bulk density of potting mix and 12 plants, half of the containers for UV-B treated plants and the other for controlled plants. Plants were treated in the field with UV irradiation (λmax = 313 nm, 30 W, lamp-leaf distance = 60 cm, 40 min-day−1). The UV-B irradiation intensity (33.5 µW/cm²) was monitored by a UV light meter (UV340B, Xin Bao Technologies, ShenZhen, China). That is, the irradiation doses of UV-B treated group was 804 J m⁻², the dose chosen was based on our previous study [57]. Control plants were maintained in the previous environment. Sampling was performed for both plants treated with UV
and controlled plants at 10 days after treatment. Morphological indicators (plant height, fresh weight of root and whole plant, leaf area) were recorded for 20 biologic repetitions, firstly. Then, these plants were sampled. Collected plants were rinsed briefly in deionized water and separated into roots, stems, leaves and petioles. The samples were then immediately frozen in liquid nitrogen and stored at −80 °C for biochemical analysis. Six biological replicates were prepared for each sample. The analysis of H$_2$O$_2$ and antioxidant enzymes was performed based on the methods of Liu et al. [59–61]. Chlorophyll analysis was performed in accordance with the methods of Arnon [62,63].

2.2. Primary Metabolite Extraction and Gas Chromatography-Mass Spectrometry Analysis

Plant samples were pulverized to 5 μm using a grinding instrument (MM 400, Retsch GmbH, Haan, Germany), and 90 mg aliquots of roots, stems, leaves, and petioles were extracted. A mixed solution of 40 μL 2-chloro-L-phenylalanine (0.3 mg/mL, internal standard) in 360 μL methanol (pre-cooled at −20 °C) was prepared for metabolite extraction. Metabolites were extracted with the mixed solution by sonication for 30 min, after which they were sonicated again with a chloroform and water solution. Subsequently, the solution was centrifuged at 14,000 rpm (revolutions per minute). The supernatant was collected in a derivatized glass bottle, evaporated to dryness. A derivatization method oximation was performed using a UPLC/Q-TOF-MS system (Waters). The MS conditions were set as follows: positive ion mode, capillary voltage of 3.0 kV, cone voltage of 45 V, source temperature of 400 °C, desolvation temperature of 500 °C, cone gas flow of 50 L/h, and desolvation gas flow of 800 L/h. Detection was performed in positive ion mode in the m/z range of 50–1000.

2.3. Phenolic Metabolite Extraction and Liquid Chromatography-Mass Spectrometry Targeted Analysis

Plant samples were pulverized to 5 μm using a grinding instrument (MM 400, Retsch GmbH), and 500 mg aliquots of roots, stems, leaves, and petioles were extracted. Samples were extracted with 80% ethanol in water (10 mL) containing 0.1 mg/L lidocaine (internal standard) by sonication twice, each for 45 min. The sample extractions were filtered, and the filtrates were merged. The filtrates were then dried under low pressure using a vacuum cavitation instrument. The resultant extracted material was dissolved in the mobile phase (1 mL) and filtered through 0.22 μm-diameter micropores. The purified solution was analyzed by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS).

Separation was performed on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 50 mm) with a VanGuard precolumn (BEH C18, 1.7 μm, 2.1 × 5 mm; Waters, Shang-Hai, China) and maintained at 30 °C. The volume injected was 2 μL. Gradient elution was performed at a flow rate of 0.25 mL·min$^{-1}$ using the following solvent system: 0.05% acetic acid-water (A), 0.05% acetic acid-acetonitrile (B); 5% B-95% B from 0–23 min; 95% B-5% B from 23–25 min; and 5% B from 25–31 min. Analyses were performed using a UPLC/Q-TOF-MS system (Waters). The MS conditions were set as follows: positive ion mode, capillary voltage of 3.0 kV, cone voltage of 45 V, source temperature of 400 °C, desolvation temperature of 500 °C, cone gas flow of 50 L/h, and desolvation gas flow of 800 L/h. Detection was performed in positive ion mode in the m/z range of 50–1000.
2.4. Multivariate Analysis

The GC-MS raw data were transformed into NetCDF format by data analysis software (Agilent GC-MS 5975) and were later processed using the software R. Each compound was displayed as the peak area normalized to that of the internal standard. For further analysis, the treated R output data were exported to Microsoft Excel. National Institute of Standards and Technology (NIST) 14 library was searched to compare the structures of the compounds with that of the NIST database. Compounds were then identified based on the retention index and mass spectra with already known compounds in the NIST library. Peak detection, retention time alignment, and library matching were performed using the TargetSearch package from Bioconductor (Solvusoft Corporation, Los Angeles, CA, USA) [64], after which the normalized data were imported into SIMCA-P version 11.0 software (Umetrics, Umeå, Sweden) for multivariate statistical analysis. The supervised method of partial least-squares discriminant analysis (PLS-DA) was used to compare tissue-specific differences between control and UV-B treatment regarding the identification of significant metabolites, and t-test combinatorial approaches were used to screen for important metabolites ($p < 0.05$). The LC-MS data were analyzed using the software MassLynx version 4.1. This software detected peaks and listed the detected and matched peaks with the retention time and $m/z$ pair and their corresponding intensities. The relative signal intensities of compounds were standardized by dividing them by the intensities of internal standard. The relationships between 15 primary metabolites and phenolic compounds were used for hierarchical clustering analysis (HCA) by R (www.r-project.org) for both species. Pearson’s correlation coefficients were calculated for these metabolites, and the Tukey test was performed using Statistical Product and Service Solutions (SPSS) version 17.0. Metabolic pathways were analyzed using the Metaboanalyst web portal (www.metaboanalyst.ca) and MBRole (http://csbg.cnb.csic.es/mbrole). The pathways of metabolites were analyzed using database sources including the Kyoto Encyclopedia of Genomes and Genomes (KEGG) (http://www.genome.jp/kegg/) to identify the most affected metabolic pathways and facilitate further metabolite interpretation. The metabolites and corresponding pathways were imported into Cytoscape software version 3.1.0 to visualize the network models. A metabolic correlation distribution network was created from the 144 primary metabolite data using the WGCNA package (Solvusoft Corporation, Los Angeles, CA, USA) [65,66].

2.5. RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

The extraction and derivatization of RNA from roots, leaves, stems and petioles were performed as described previously by Liu et al. [67]. Amplification, detection, and data analysis were performed using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). Primer sequences for PCR were as follows (Table 1):

| Gene Name | Primer Sequence (5' to 3') |
|-----------|---------------------------|
| CHS       | Forward: CTTTCTTGGATGCTAGACAAGACA, Reverse: CGAAGACCAAGAGTTTGGTTAGTT |
| PAL       | Forward: CATCAAATCTCTTGAGCAGTGGAA, Reverse: AGTTCACATCTTGGTTATGCTGCTC |
| C4H       | Forward: AAAAAAGTGAGGGATGAAATTGACA, Reverse: GGATTGCCATTCTTGCAGC |
| 4CL       | Forward: TGTCCTTCTCTGGTTATGCTGTC, Reverse: CTTTGGGGAATTTAGCTCTGACAGT |
| CHR       | Forward: AAACAGGTTACAGGCATTGTTGACA, Reverse: GGAAGAACGAGATGAGGATGATT |
Table 1. **Cont.**

| Gene Name | Primer Sequence (5' to 3') |
|-----------|----------------------------|
| CHI       | ATCGAGTTTTTCCACCAGGATCTAC  |
| Reverse   | ATCATAGTCTCAACACAGCCTCAG   |
| IFS       | CCTTCACATATTGGACAAAACCTCTT |
| Reverse   | CCTGTATTAAAAAGGAAGAGCCTCA  |
| IOMT      | GCACAAAAACACAGATCAAACTTC   |
| Reverse   | GCATTACGGCCATTGATTG        |
| 13'H      | GATGTTAAAAAGCGAAGCAATTT    |
| Reverse   | ATCAAAACAATCTCAACAAAGGCAA |
| UCGT      | AGGTTTGGAAGATTATGCACCA     |
| Reverse   | TCCCTTCTGAGTCCAGGACA       |
| 18S       | TGCAGATCCCCGTGAACCATC      |
| Reverse   | AGGCATCGGGCAACGATATG       |

To determine relative fold differences in template abundance for each sample, the cycle threshold (Ct) values for each of the gene-specific primers were normalized to the Ct value for 18S RNA.

3. Results

3.1. **Morphological and Physiological Changes Induced by Ultraviolet-B Radiation**

The effect of UV-B radiation on morphology can be seen in Figure 1 and Table 2. Compared with the control group, stem growth was relatively short and the leaf more compact under UV-B radiation. Both plant height and leaf area decreased under UV irradiation. The whole-plant proportion of roots ($n = 10$) was 19.07% for the control group and 20.70% for the UV-B treatment group. In contrast to the control group, the chlorophyll content decreased in the UV-B treated group. The activity of antioxidant enzymes and content of $H_2O_2$ increased under UV-B radiation.

**Table 2.** Morphological and physiological indices of control and Ultraviolet treatments ($n = 20$).

|                | Height (cm) | Root/Whole Fresh Weight (FW) (%) | Leaf Area (cm²) | Chlorophyll (mg g⁻¹) |
|----------------|-------------|----------------------------------|-----------------|----------------------|
| Control        | 24.2 ± 0.6  | 19.07 ± 0.31                     | 0.292 ± 0.004   | 1.242 ± 0.008        |
| UV-treated     | 23.4 ± 0.6  | 20.7 ± 0.67                      | 0.230 ± 0.006   | 0.799 ± 0.011        |

|                | CAT (U g⁻¹·min⁻¹, FW) | POD (U g⁻¹·min⁻¹, FW) | APX (U g⁻¹·min⁻¹, FW) | $H_2O_2$ (µmol·g⁻¹) |
|----------------|------------------------|------------------------|------------------------|---------------------|
| Control        | 26.25 ± 0.01           | 1800 ± 1               | 0.06 ± 0.001           | 3.59 ± 0.01         |
| UV-treated     | 36.75 ± 0.01           | 7050 ± 1               | 0.15 ± 0.003           | 3.79 ± 0.01         |

Abbreviations: CAT: catalase; POD: peroxidase; APX: ascorbate peroxidase; $H_2O_2$: hydrogen peroxide.
Figure 1. Metabolic analysis of specific tissues of *A. mongholicus* under control and UV-B treatments analyzed by GC-MS. (A) Partial least-squares discriminant analysis (PLS-DA); (B) The expression pattern of potential differences. a, mannose; b, 3-mannobiose; c, fructose; d, talose; e, glucose; f, psicose; g, sorbofuranose; h, myo-inositol; i, 2,3,4-trihydroxybutyric acid; j, 4-aminobutyric acid; k, citric acid; l, hexonic acid; m, isothiocyanic acid; n, malic acid; o, oxalic acid; p, phosphonic acid; q, palmitic acid; r, propanedioic acid; s, stearic acid; t, pinitol; u, erythritol; w, D-glucopyranoside; x, 2-O-glycerol-D-galactopyranoside; y, methyl galactoside; z, glycerol; ab, dotriacontane; ac, 1-monopalmitin.
3.2. Primary Metabolism Reprogramming between the below- and Aboveground Organs in Response to Ultraviolet-B Radiation

In the present study, approximately 184 metabolites comprising sugars, acids, alcohols, and other compounds were identified using a GC-MS platform in four tissues of *A. mongholicus* under control and UV-B treatments (Supplementary Table S1).

To identify the metabolites that contributed to these variances, 27 significantly differential compounds were identified by their variables of importance in the projection (VIP) values >1, and their *p*-values <0.05 (Figure 1B). There were eight significant differences regarding sugars; most of the sugars were monosaccharides. The 3-α-mannobiose was the only disaccharide that significantly changed under UV-B, and its monosaccharide (mannose) constituents have a common tendency to decrease in aboveground tissue (stems and petioles). In contrast to the control, the levels of d-glucose and myo-inositol increased in roots and stems but decreased in leaves and petioles under UV-B treatment. The d-talose was significantly reduced by UV-B radiation in belowground tissues (roots) but did not significantly change in aboveground tissues. Regarding acids, including eight organic acids, one inorganic acid and two fatty acids, diverse trends were observed. Phosphonic acid level decreased under UV-B radiation in all organs. Propanedioic acid increased only in roots, and there was no significant difference in aboveground tissue under control or UV-B treatments. Palmitic acid decreased in belowground tissue (roots) but increased in aboveground tissues (stems) under UV-B radiation. Oxalic acid increased in leaves and stems while hexonic acid increased in leaves and petioles under UV-B radiation. Significant increases in alcoholic compounds in belowground tissues (roots) were observed for α-pinitol, whereas significant increases in aboveground tissues (leaves) were observed for erythritol. In addition, the levels of six other compounds significantly differed from those of the control group under UV-B radiation. Among these compounds, α-α-glucopyranoside levels significantly differed only in belowground tissues (roots).

The PLS-DA was used to profile these metabolites. In the PLS-DA score plot, separation of the two treatments and their different tissues was obtained. The samples were clustered into separate groups (Figure 1A). A PLS-DA model was created with two principal components: PLS1 (47.9%) and PLS2 (64.7%). A clear classification trend was observed among roots, stems, leaves, and petioles for all samples in the score plot. The control and UV-B treatment groups of the aboveground organs (stems, leaves and petioles) were better separated than those of the belowground organs (roots).

3.3. Phenolic Compounds Were Concentrated in Leaves Compared to Roots under Ultraviolet-B Radiation

To understand the effects of increased UV-B radiation on the level of phenolic metabolites, the targeted phenolic compounds content of the collected samples was analyzed via LC-MS. A total of 29 standard reference compounds were used, all provided by Shanghai yuanye Bio-Technology Co. Ltd (ShangHai, China), with a purity of ≥98%. The targeted compounds in the samples were identified in accordance with the mass spectral information and retention time of the corresponding standard reference compounds. The mass spectral information has been added as Supplementary Table S2; the retention time and corresponding standard reference compounds were shown in the chromatogram of Figure 2. In total, 29 phenolic metabolites were analyzed.
Figure 2. Chromatogram of the target compounds from ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-TOF-MS). Targeted compounds in samples were determined in accordance with the mass spectral information and retention time of the corresponding standard reference compounds. QR: Quercetin-3-O-rhamnoside.

To further clarify phenolic accumulation patterns in different tissues under the control and UV-B treatments, the roots, stems, leaves, and petioles of *A. mongholicus* were determined separately. Visualization of the phenolic profile was performed using HCA (Figure 3). The accumulation of phenolics displayed a clear phenotypic variation in terms of phenolic abundance in the different tissues and treatments. Roots contained the highest levels of the majority of tested phenolics, followed by the leaves, petioles and stems. Based on their tissue- and UV-B specific accumulation patterns, phenolics clearly grouped into two clusters.
Figure 3. Distribution of potential biomarkers in specific tissues from the control and UV-B treatment groups. Heat map visualization of the relative differences of potential biomarkers in the different samples. The data of the content value of each compound were normalized to complete the linkage hierarchical clustering. Each tissue type is visualized in a single column, and each metabolite is represented by a single row: CK-R, CK-S, CK-L, and CK-P correspond to roots, stems, leaves, and petioles of the control group; UV-R, UV-S, UV-L, and UV-P correspond to roots, stems, leaves, and petioles from the UV-B-treated group. Red indicates high abundance of compounds, whereas green indicates low abundance (color key scale above heat map).

Phenolics in cluster I showed higher levels in the roots of the UV-B treatment group than in those of the control group and mostly consisted of calycosin-7-glucoside, ononin, formononetin, isoliquiritigenin, and liquiritigenin. Phenolics in cluster II consisted of 4-hydroxybenzoic acid, L-phenylalanine, luteolin, and myricitrin; higher levels of these compounds were detected in the leaves of the UV-B radiation group than in those of the control group. In addition, UV-B radiation altered the distribution of phenolic compounds between above- and belowground tissues (Figure 4). The ordinate of Figure 4 is set to the ratio of compound contents in leaves and roots.
According to Figure 4, the majority of the ratios of phenolic levels in leaves/roots under UV-B treatment were higher than those of the control. In other words, compared with those of the control, phenolic compounds were more concentrated in leaves under UV-B radiation. Moreover, all the C6C3 carbon skeleton phenolics were translocated from the roots to the leaves.

3.4. Construction of an Integrative View of the Primary and Phenolic Metabolite Network for Specific Tissues and Growth Conditions

To provide better overview of these data, a simplified primary and phenolic metabolites pathway was used to show the metabolic responses to UV-B radiation among different tissues. As shown in Figure 5A,B, for primary metabolites, the accumulation of soluble sugars, such as glucose, fructose, and mannose, decreased in response to UV-B radiation in both roots and leaves. In difference with leaves the level of erythritol and sorbitol increased in the roots. Some amino acids including valine and aspartate increased in response to UV-B radiation in both leaves and roots. The levels of acids involved in the tricarboxylic acid (TCA) cycle, including fumarate in roots, malate, and succinate in leaves, increased in the UV-B treatment group. For phenolic metabolites, most of the isoflavones in roots were upregulated by UV-B induction. Unlike roots, the upregulated phenolic metabolites in leaves mainly belong to phenolic acids and flavones. We only observed that the C6C3 phenolics decreased both in roots and leaves. In addition, phenylalanine, which is the key node in the synthesis of phenolic compounds from the shikimate pathway, may be responsible for the increase in the majority of phenolic compounds under UV-B radiation.

Figure 4. Distribution of phenolics that have various carbon skeletons in above- and belowground tissues under UV-B radiation. The ordinate is set to the ratio of compound contents in leaves and roots. (A) Phenolics with C6C1 carbon skeletons; (B) phenolics with C6C3 carbon skeletons; (C) and (D) phenolics with C6C3C6 carbon skeletons.

Biomolecules 2020, 9, x FOR PEER REVIEW 11 of 22

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Figure 4. Distribution of phenolics that have various carbon skeletons in above- and belowground tissues under UV-B radiation. The ordinate is set to the ratio of compound contents in leaves and roots. (A) Phenolics with C6C1 carbon skeletons; (B) phenolics with C6C3 carbon skeletons; (C) and (D) phenolics with C6C3C6 carbon skeletons.

According to Figure 4, the majority of the ratios of phenolic levels in leaves/roots under UV-B treatment were higher than those of the control. In other words, compared with those of the control, phenolic compounds were more concentrated in leaves under UV-B radiation. Moreover, all the C6C3 carbon skeleton phenolics were translocated from the roots to the leaves.

3.4. Construction of an Integrative View of the Primary and Phenolic Metabolite Network for Specific Tissues and Growth Conditions

To provide better overview of these data, a simplified primary and phenolic metabolites pathway was used to show the metabolic responses to UV-B radiation among different tissues. As shown in Figure 5A,B, for primary metabolites, the accumulation of soluble sugars, such as glucose, fructose, and mannose, decreased in response to UV-B radiation in both roots and leaves. In difference with leaves the level of erythritol and sorbitol increased in the roots. Some amino acids including valine and aspartate increased in response to UV-B radiation in both leaves and roots. The levels of acids involved in the tricarboxylic acid (TCA) cycle, including fumarate in roots, malate, and succinate in leaves, increased in the UV-B treatment group. For phenolic metabolites, most of the isoflavones in roots were upregulated by UV-B induction. Unlike roots, the upregulated phenolic metabolites in leaves mainly belong to phenolic acids and flavones. We only observed that the C6C3 phenolics decreased both in roots and leaves. In addition, phenylalanine, which is the key node in the synthesis of phenolic compounds from the shikimate pathway, may be responsible for the increase in the majority of phenolic compounds under UV-B radiation.
Figure 5. Cont.
Figure 5. Visualization of primary and phenolic metabolites dynamics on a biochemical pathway map. (A) Metabolism map in roots. (B) Metabolism map in leaves. Compounds in the red box are upregulated by UV-B radiation, and compounds in the green box are downregulated.
3.5. Expression of Genes Involved in Isoflavonoids Pathway in Leaves and Roots

To further explore the metabolic mechanisms of CAG in the enhanced UV-B environment, the CAG biosynthesis pathway and the expression levels of relevant genes were visualized, as shown in Figure 6. The expression of the genes encoding enzymes that directly participate in the formation of CAG in samples collected from different treatments and different tissues was analyzed using RT-PCR. Gene expression levels were normalized using the 18S RNA reference gene as an internal standard. The transcription level of the synthetase genes involved in the CAG pathway, including CHI, IFS, IOMT, I3'H, and UCGT, were upregulated in roots but downregulated in aboveground tissues in response to UV-B induction. This variation is strikingly similar to the accumulation pattern of their corresponding compounds. The CHS and CHR co-catalyze the condensation of p-coumaryl-CoA with three malonyl-CoA molecules toward the formation of the isoliquiritigenin, an isoflavonoid skeleton. The increased expression of CHS was induced by UV-B in the roots. Compared with CHR, CHS is clearly more responsive to UV-B.

Figure 6. Visualization of isoflavonoid metabolites and relative gene dynamics on a biochemical pathway map. The levels of isoflavonoid metabolites and relative genes were both averaged over three biological replicates after normalization. Within each box, each column is a specific tissue (from left to right: root, R; stem, S; leaf, L; petiole, P), as shown in the upper-right corner. The intensity of the ratio of the UV-B group to the control (CK) group is indicated by the color scale key in the upper-right corner.
4. Discussion

Increased UV-B radiation affects plant development, morphology, physiology and metabolism. At the same time, environmental vicissitude will greatly impact A. mongholicus in the field. Therefore, the metabolic response of A. mongholicus to UV-B strongly influences the quality of Radix astragali that is provided to the market. In this study, we used untargeted GC-MS and targeted LC-MS techniques at primary and secondary compound levels to investigate the abundance and identity of compounds, revealing different metabolic profiles for specific tissues under different conditions. To explore the reprogramming of primary and secondary metabolic responses to UV-B and the formation of adaptive stable metabolism in above- and under-ground tissues. We also evaluated the level of isoflavones and their related genes to explore the mechanism of CAG accumulation under UV-B.

Based on the observation of morphological indicators and the detection of physiological indicators (Figure 1 and Table 1), leaves were more compact and plant height, leaf area, and chlorophyll content were reduced. These results indicate that growth was inhibited and that energy might be transferred to the accumulation of more functional metabolites to adapt to the changing environment [68]. The relative root biomass increased slightly, suggesting that energy was transferred to the roots in response to the UV-B radiation. Moreover, an increase antioxidant enzymes and H₂O₂ (Table 1) indicated that the stress response of plants to UV-B was actively operating [69,70].

We based our study on GC-MS-metabolomics to analyze tissue-specific changes under UV-B radiation and control conditions of A. mongholicus. Sugars, which are energy sources for growth, decreased in aboveground tissues but increased in belowground tissues. This explains the decrease in plant height and leaf area and the increase in root specific gravity. Additionally, the reduction in glucose in the leaves could indicate that a stronger shift from carbon assimilation to carbon accumulation occurred after UV-B radiation. Moreover, increased levels of sugar alcohols (e.g., erythritol) derived from the pentose phosphate pathway (PPP) were also observed in the UV-B treatment group, likely reflecting the higher respiratory rate of the PPP pathway in these plants. In addition, PLS-DA plots of the primary metabolites for four tissues under different conditions were generated (Figure 1A). The analysis of the score plots revealed a clear clustering of biological replicates for each sample.

The dynamic patterns of targeted phenolic compounds in four tissues of control and UV-B treatment plants was detected by UPLC-q/TOF-MS. Interestingly, the content of flavanones and flavonols increased in the leaves in response to UV-B radiation, whereas the isoflavonoids increased in the roots. Several early physiological experiments provided circumstantial evidence that phenolics are involved in UV-B protection [71]. Among these phenolics, flavones and flavonols protect cells because these compounds accumulate in the epidermal layers of leaves and stems, acting as filters and absorbing radiation in the UV-B portion of the spectrum [72,73]. Ryan et al. reported that UV radiation induces the synthesis of flavonols that have higher hydroxylation levels in Petunia and Arabidopsis. Those authors suggested that flavonols may play as yet uncharacterized roles in the UV stress response because flavonols have UV-absorbing properties, and the hydroxylation of flavonols might positively affect antioxidant capacity [74,75]. Regarding compounds involved in UV-B protection, the structures of flavones and flavonols are the most advantageous [15]. Thus, the most significant changes occurred regarding the levels of flavone and flavonol compounds (such as luteolin and myricitrin) under UV-B radiation were in leaves. At the same time, the accumulation of isoflavonoids (such as calycosin-7-glucoside, ononin, and formononetin) in roots constitutes a reserve of active phenolics that can easily be mobilized at any given time, especially under UV-B stress conditions. Furthermore, a common feature of the changes in phenolic compounds under UV-B exposure in roots and leaves was observed. The C6C3C6 carbon compounds increasingly accumulated at the expense of C6C3 carbon compounds such as chlorogenic acids, ferulic acid, and cinnamic acid. In view of this phenomenon, the increase in C6C3C6 carbon compounds (flavone and flavonol in the leaves and isoflavonoid in the roots) at the cost of C6C3 carbon compounds may be due to the stronger UV-B absorptive and antioxidant capacity of C6C3C6 carbon compounds than that of C6C3 ones in A. mongholicus. Under UV-B radiation, the phenolic compounds of various carbon skeletons are
concentrated in the leaves (Figure 4), probably because the leaves are most directly affected by UV-B radiation. The decreasing of sugars and enrichment of phenolics in leaves could also result in reduced energy for growth and reduced leaf area.

To obtain a more detailed overview regarding the tissue-specific differences of the identified metabolites between the UV-B and control treatments, we built a primary and phenolic metabolites network (Figure 5A,B). The TCA cycle intermediates such as succinate and fumarate, which are major regulators of carbon and nitrogen interactions, increased under UV-B radiation. At the same time, this increase also occurred for shikimate and L-phenylalanine. It seems highly likely that shikimate would be elevated following the mobilization of carbon reserves stored in plants to increase the flux through the phenylpropanoid pathway under UV-B radiation [76]. In plants, phenylalanine is thought to be the general precursor of C6C1-, C6C3-, and C6C3C6 compounds and their polymers such as tannins and lignins [77]. As such, when considered together, these data suggest that in response to UV-B the plant cell is “primed” at the level of primary metabolism by a mechanism that involves the reprogramming of metabolism to efficiently divert carbon toward the aromatic amino acid precursors of the phenylpropanoid pathway [78]. Tryptophan and phenylalanine compete for chorismate to synthesize alkaloids and phenols, respectively. The results showed more accumulation of phenylalanine under UV-B radiation. This suggests that the increase in phenolic compounds under UV-B radiation is caused by the transfer of carbon from primary metabolism and involves metabolic reprogramming.

For phenylpropanoid pathway under UV-B radiation, a significant difference appears between the above- and the underground tissues. Flavones were significantly increased in leaves, probably because of the roles of flavones accumulation in the epidermal layers of leaves acting as filters and absorbing radiation in the UV-B portion of the spectrum [79]. The phenolic acids enhanced accumulation in leaves might be acting as antioxidant supplements. Different from the metabolic response in leaves, enhanced accumulation of isoflavones was observed in roots. These isoflavones might constitute a reserve of active phenolics that can easily be mobilized at any given time, especially under UV-B stress conditions. Phenylalanine, as a key node in phenolic metabolism, showed a significant increase only in leaves under UV-B radiation. A bold and reasonable assumption is that the upregulation of the relevant genes rather than the synthetic precursor (phenylalanine) might be the most important contributor for the activation of phenolic metabolism in the roots.

In order to verify the possibility of this assumption for further, and due to the therapeutic potential of these isoflavones (especially CAG), the metabolic mechanisms of these compounds in the enhanced UV-B environment need to be further explored. The CAG biosynthesis pathway and relevant genes are visualized in Figure 6. The promoters of a series of genes involved in the flavonoid biosynthetic pathway contain a specific recognized domain that can interact with the MYB family of transcription factors via light-responsive elements [80]. In the present study, targeted flavonoid biosynthetic genes have transcriptional activation; as suggested, these transcription factors could be activated by UV-B radiation and could affect the investigated genes (Figure 6). In general, PAL genes have been proposed as a dominant control point of phenylpropanoids, flavonoids, and isoflavonoids biosynthesis in response to various biotic and abiotic stresses inclusive of pathogen attack, UV radiation, and mechanical wounding [81,82]. The no-significant of phenylalanine and increased transcription levels of PAL indicates that the response of phenolics in roots to UV-B is activated by relevant genes rather than phenylalanine. The increased transcription levels of PAL in roots, stems, and leaves indicated that the response of phenolics to UV-B is stimulated in different tissues. CHS and CHR co-catalyze the condensation of p-coumaryl-CoA with three malonyl-CoA molecules toward the formation of the Isoliquiritigenin, an isoflavonoid skeleton. The increased expression of CHS was induced by UV-B in the roots. Compared with CHR, CHS is clearly more responsive to UV-B, which suggests that the increased accumulation of isoflavonoids might be due to the elevated levels of CHS in the UV-B environment. The transcription level of the synthetase genes involved the CAG pathway, including CHI, IFS, IOMT, I3′H, and UCGT, were upregulated in the roots in response to UV-B induction but downregulated in aboveground tissues. This variation is strikingly similar to the accumulation pattern
of the corresponding compounds of these enzymes. These results suggest that in the enhanced UV-B environment, tissue-specific increases in the levels of active isoflavones such as CAG are due to the regulation of the elevated levels of synthesis genes in the roots.

5. Conclusions

A metabolic profile was revealed using untargeted GC-MS and targeted LC-MS combined techniques to investigate the tissue-specific metabolic mechanism of A. mongholicus in an enhanced UV-B environment. We found that in response to UV-B the plant cell is “primed” at the level of primary metabolism by a mechanism that involves the reprogramming of metabolism to efficiently divert carbon toward the aromatic amino acid precursors of the phenylpropanoid pathway. A stronger shift from carbon assimilation to carbon accumulation has occurred. Among the accumulation of phenolics, C6C1 carbon compounds (phenolic acids in leaves) and C6C3C6 carbon compounds (flavones and flavonols in leaves and isoflavones in roots) increased at the expense of C6C3 carbon compounds in order to obtain the stronger UV-B absorptive and antioxidant capacity. Compared with the control treatment, the response of phenolics has a significant tissue-specific in the UV-B radiation treatment. Notably, the response of phenolics in roots to UV-B is activated by upregulation of relevant genes rather than phenylalanine.

Supplementary Materials: The following are available online at http://www.mdpi.com/2218-273X/10/4/504/s1, Table S1: Compounds identified in Astragalus mongholicus measured by Gas Chromatography-Mass Spectrometry; Table S2: Metabolite reporting checklist and recommendations for Liquid Chromatography-Mass Spectrometry. 

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