Differential Expression of Calycosin-7-O-β-D-glucoside Biosynthesis Genes and Accumulation of Related Metabolites in Different Organs of Astragalus membranaceus Bge.var. mongholicus (Bge.) Hsiao Under Drought Stress

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Research Article

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Differential Expression of Calycosin-7-*O*-*β*-D-glucoside Biosynthesis Genes and Accumulation of Related metabolites in Different organs of *Astragalus membranaceus* Bge.var. *mongholicus* (Bge.) Hsiao Under Drought Stress

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Abstract

Flavonoids are secondary metabolites that accumulate in most plants. Calycosin-7-*O*-*β*-D-glycoside (CG), as a flavonoid, plays an important role in the abiotic stress response of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao (*A. mongholicus*). CG is also an active ingredient in *A. mongholicus* with high medicinal value. However, the response mechanism of CG biosynthetic pathway to drought stress is not clear. In this research, drought stress was inflicted upon annual potted *A. mongholicus* for 15 days and the variations in flavonoid metabolites and the correlating gene expression in CG biosynthesis were studied in roots, stems and leaves of *A. mongholicus* by UHPLC-MRM-MS/MS and qRT-PCR. Drought stress reduced the dry weight and increased the content of MDA and Proline. Drought promoted the accumulation of most compounds in the CG synthetic pathway of *A. mongholicus*, which decreased after rewatering. *AmI3'H* is highly expressed in the roots under drought stress. Overexpression of *AmIOMT* was observed in the leaves, but the content of formononetin which is the product of IOMT catalysis was higher in stems than in leaves. This research aims to further understand the acclimation of plant to abiotic stress and the regulation mechanism of flavonoid accumulation in astragalus species.

Keywords: Drought stress *A. mongholicus* Secondary metabolite qRT-PCR Targeted metabolome

Introduction

Secondary metabolites are an important group of compounds essential for plant acclimation and survival to varying environmental conditions, which can be classified into three major types, including terpenoids, nitrogen-containing metabolites and phenolics[1]. As antioxidants, phenolic compounds (tannins, flavonoids and lignin) can inhibit the generation of reactive oxygen species (ROS)[2] and contribute to counteracting the negative impacts of drought stress[3]. The main reason for drought-induced accumulation of phenolic acids and flavonoids is the modulation of phenylpropanoid biosynthetic pathway[4]. Drought enhanced the transcription levels of PAL, C4H and F3H, which are genes encoding key enzyme in enzyme-catalyzed phenylpropanoid biosynthesis, leading to increase in the flavonoid levels[5, 6]. In soybeans, flavonoid biosynthesis is upregulated under drought-induced oxidative stress [7]. Controlled drought stress significantly increased the production of glycyrrhizic acid and liquiritin without reducing root biomass [8]. Drought stress enhanced beta caotene composition in *Choysum varieties*[9], the content of total antioxidant activity, total polyphenols and total flavonoids content in *Silybum marianum* [10], *Achillea* species [11], *Amaranthus tricolor* [12, 13]. Successful and effective use of deliberate drought stress can directly increase the production of secondary metabolites, which can be achieved by applying simple and inexpensive special irrigation systems [14].

Radix astragali is the dried root of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) (*A. mongholicus* or *Astragalus membranaceus* (Fisch.) Bge.). It is one of the most commonly employed natural herbs in China, Japan, Korea and other Asian regions. Currently, the natural

resources of *A. mongholicus* is dwindling and commercial supply mostly depends on artificial cultivation. The quality of radix astragali is related to the content of biological active compounds. While calycosin-7-*O*-*β*-D-glucoside (CG) is one of them, which plays an important role in preventing osteoporosis [15], relieving myocardial injury upon hyperthermia [16], antioxidant [17], and anticancer [18].

The three early steps of the conversion from phenylalanine to cinnamic acid derivatives in the biosynthetic pathway of flavonoids are identical for all major phenylpropane pathways. Therefore, these series of reactions are known as 'generic phenylpropane metabolism' [19]. As shown in Fig. 1[20], PAL, the first enzyme to enter the flavonoid pathway [21], converts the L-phenylalanine to cinnamic acid, Then C4H [22] and 4CL were applied in cinnamic acid to form 4-coumaroyl-CoA [23]. The first step during the biosynthesis of flavonoids and flavonol glycosides is catalyzed by chalcone synthase (CHS), which is a key enzyme in various flavonoid pathways [24]. In the following reaction, naringin (5,7,4-trihydroxy flavonoids) was generated by isomerization of chalcone isomerase (CHI) [25]. During the synthesis of isoflavone species, isoflavone synthetase (IFS) catalyzes the formation of all isoflavones [26] ,which has the function of converting naringin into genistein and catalyzing daidzein. A methyl group is then added in daidzein to form formononetin by isoflavone O-methyltransferase (IOMT) [27]. Finally, isoflavone 3' -hydroxylase (I3'H) is hydroxylated to form calycosin, and the compound can be modified into CG by UDP-glucose: calycosin-7-*O*-glucosyl transferase (UCGT) [20].

Our previous studies have shown that moderate drought is conducive to the accumulation of

CG[28]. Therefore, a possibility is that the use of water stress to improve the quality of *A. mongholicus*. The premise of achieving this goal is to grasp the response to drought stress of *A. mongholicus*. In this paper, changes of CG synthesis related to metabolites and enzyme gene expression levels were studied in different organs of *A. mongholicus* under drought stress, which can contribute to the further understanding of the calycosin-7-*O*-*β*-D-glucoside synthetic pathway.

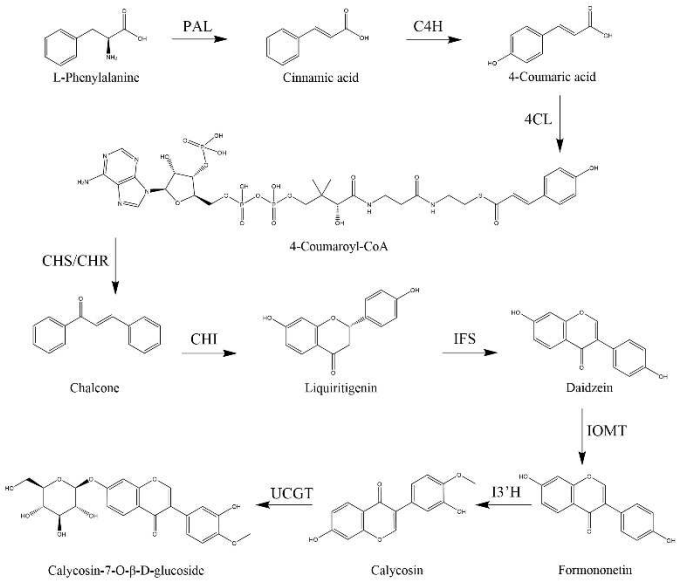


Fig. 1 Proposed calycosin and CG biosynthetic pathways in plants [18]. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl CoA ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; IOMT, isoflavone *O*-methyltransferase; I3′H, isoflavone3′-hydroxylase; UCGT, UDP-glucose: calycosin-7-*O*-glucosyltransferase.

Materials & Methods

Experimental Materials and Experimental Design

Greenhouse pot experiment was conducted in Inner Mongolia University south campus (40°17′

N, 111°38′ E, altitude: 1050 m), Hohhot city, Inner Mongolia Province, Northwest China. In the natural photon-cycle (about 14/10 h light/dark cycle) and 26±4℃/16±3℃ day/night cycle from march to June 2018. Seeds of *A. mongholicus* were purchased from Inner Mongolia Tianchuang Pharmaceutical Technology Co., Ltd. The seeds were immersed in water at 100 °C for 90 s, followed by soaking in water at 30 °C for 3 h, which were then sown in plastic pots (17 cm in diameter and 25 cm in height), 40 per pot on March 15, 2018. Cultivated soil from field soil Wuchuan County, is chestnut soil. The soil organic matter content is 12.92±1.06 g/kg, total nitrogen is 0.86±0.07 g/kg, alkali nitrogen is 53.67±10.69mg/kg, total phosphorus is 0.36±0.07 g/kg, available phosphorus is 43.77±0.93mg/kg, total potassium. 10.77±0.87g/kg, available potassium 24.25±3.55mg/kg, pH=8.14±0.03. Water is poured once every 4-5 days after the seeds are germinated. When the plant height of most seedlings was about 15cm, seedlings with roughly equal height were selected and randomly divided into two groups. Adequate water was continued to be retained in the control group, while the dry group was fully irrigated, which was then kept drought for 15 days. The roots, stems and leaves of seedlings in two groups (6 pots each) were collected as samples at 8:00 a.m. The samples were immediately frozen in liquid nitrogen and stored at -80 °C. The remaining seedlings were replenished on day 15 after drought stress. Two groups of seedlings (6 pots each) were collected at 8:00 am on day 21. Roots, stems and leaves were frozen in liquid nitrogen and placed at -80 °C.

Determination of soil water content (SWC) and root, shoot dry weight

Soil samples from six pots were collected shortly after sampling. Soil samples were dried at 105℃

for 48 h in oven to determine the DW after determination of the FW. SWC was calculated as follows: SWC (%) = [(FW - DW)/FW] ×100. Root and shoot of the seedlings were separated, which were then washed with distilled water, gently blotted dry with a filter paper, weighed for FW and then were oven dried at 60 ℃ for 48 h for DW determination, respectively.

Determination of MDA, Pro

Content of malondialdehyde (MDA), the final product of lipid peroxidation, was measured as described by Du and Bramlage [29]. Proline content was determined according to ninhydrin coloring method [30].

RNA Extraction and Real-Time PCR

The extraction of total RNA and the reaction of polymerase chain were performed based on the reference [31]. The extraction of total RNA and the reaction of polymerase chain were performed based on the reference [32].The response consisted of three biological replications, the use of *18S* as a reference was repeated to calculate the expression of each gene in a relatively quantitative manner in three techniques.

Isoflavone Extraction and UHPLC-MRM-MS/MS Analysis

This part of the experiment was commissioned by Shanghai BioTree biotech Co.,Ltd . The extraction of related compounds follows previous studies [33]. The instrument model is Agilent 1290 Infinity II series (Agilent Technologies), the target compound was chromatographed using a Waters ACQUITY UPLC HSS T3 (100×2.1 mm, 1.8 μm, Waters) liquid chromatography column. The liquid phase A phase is 0.1% aqueous acetic acid, B phase is methanol. The oven temperature

was 40 °C, the sample tray was set to 4 °C, and the injection volume was 3 μL.

Mass spectrometry was conducted in a multiple reaction monitoring (MRM) mode using an Agilent 6460 triple quadrupole mass spectrometer equipped with an AJS-ESI ion source. The ion source parameters are as follows: Capillary voltage = +4000/-3500 V, Nozzle voltage = +500/-500 V, gas (N_2) temperature = 300 °C, Gas (N_2) flow = 5 L/min, sheath gas (N_2) temperature = 250 °C, sheath gas flow = 11 L/min, nebulizer = 45 psi.

Statistical Analysis

SigmaPlot mapping was employed. Each result shown in the figure was the mean of three replicated treatments. The significant differences between treatments were statistically evaluated by standard deviation. Processing data with SPSS 19. Treatment means were compared using Duncan's multiple range examination.

Results

Soil water content (SWC) and root, shoot dry weight

The seedlings of *A. mongholicus* were treated with drought for 15 days. First, the relative water content of the soil was examined under drought stress. SWC significantly reduced from 34.17% to 22.55% on day 3, and then gradually reduced to 7.55% on day 20 (Fig.2a). The root dry weight is 0.09 g/plant, which is about 16.45% lower than the control. As a result, about half of the drought-stressed plants exhibited leaf yellowing and curling. The shoot dry weight was 0.20 g/plant, which was decreased by 33.56% compared with the control. Root dry weight decreased significantly to be about 0.09 g/plant on day 15, which was 83.6% of the control. Shoot dry weight continued to

decrease to be about 0.20 g/plant on day 3, which was 66.4% of the control (Fig.2b).

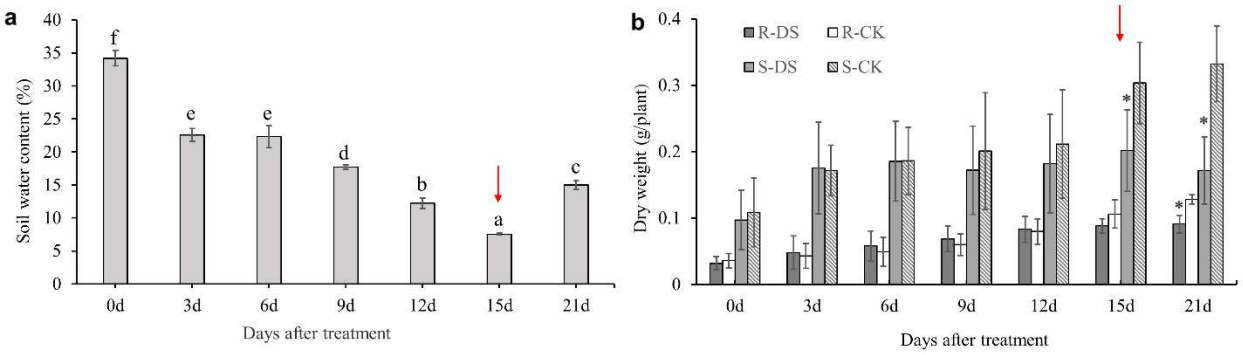


Fig.2 Effects of progressive drought stress and rehydration on soil water content (a), plant growth variables(b) R-DS: root dry weight under drought stress, R-CK: root dry weight under control conditions, S-DS: shoot dry weight under drought stress, S-CK: shoot dry weight under control conditions. Red arrows indicate to start rehydration at the date. Data represent the mean values ± SE. The asterisk represents significant difference ($P < 0.05$)

Physiological changes during drought acclimation

MDA is one of the lipid peroxidation products of plant cell membranes. The MDA contents in roots, stems and leaves are is increased under drought stress. The MDA content in roots increased significantly from day 9, it reached 3.54 times of the control by day 12.MDA levels in stems and leaves increased by 52% and 66% respectively (Fig. 3a) on day 14. The content of proline in roots, stems and leaves increased with the prolonged drought. The proline content increased slowly at the beginning of the drought and increased remarkably on day 12. On day 15, the proline contents in leaves, stems and roots were 34.77, 54.27, 36.29 times of that on day 0 (Fig. 3b).

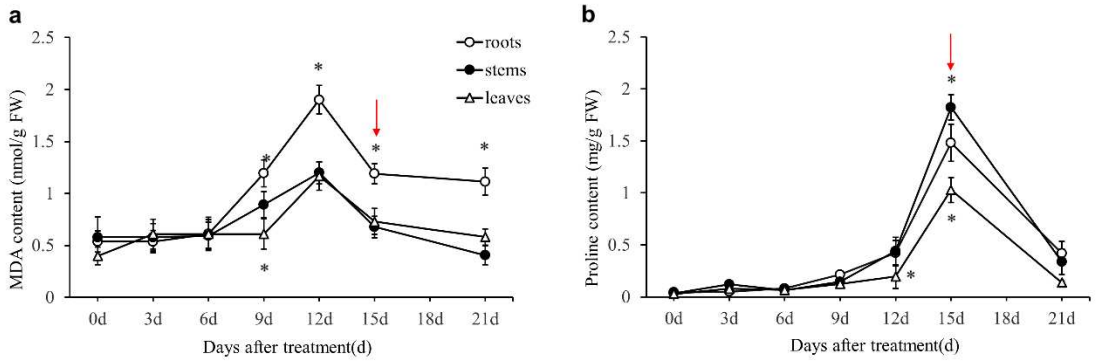


Fig.3 Changes of *A. mongholicus* Physiological Indexes under Drought Stress (a) MDA content (b) proline contents. Red arrows indicate to start rehydration at the date. Data represent the mean values ± SE. The asterisk represents significant difference ($P < 0.05$)

The Expression Levels of Related Genes in Different Organs of *A. mongholicus* Under Drought Stress

To study the related genes in calycosin and CG biosynthesis, the expression levels of genes related to CG biosynthesis in root, stem, and leaf are investigated using qRT-PCR. The expression levels of *AmIOMT*, *AmIOMT*, *AmCHR*, *AmIFS*, *AmI3'H*, *AmPAL, AmC4H*, *Am4CL* and *AmCHI* are expressed in Fig. 4. The expression level of *AmIOMT* in leaves increased significantly under drought treatment. The expression level of *AmCHS* in stems was significantly increased. The expression levels of *AmCHR*, *AmIFS* and *AmI3'H* in the roots increased significantly (Fig. 4). The expression levels of *AmPAL*, *AmC4H* and *Am4CL* showed similar expression patterns under drought stress, wherein, expression level of *AmPAL* was increased by about three-fold in the root. PAL, CHI and IFS, catalytic synthesis of respective cinnamic acid, liquiritigenin and daidzein in the CG pathway, was upward expression levels in roots of *A. mongholicus* under drought treatment,

just as the trends of these three compounds. The expression levels of *AmCHR*, *AmCHI*, *AmIFS* and *AmI3'H* in roots were the highest, while the expression levels of *AmCHR*, *AmCHI*, *AmIFS* and *AmI3'H* in leaves were the lowest. The expression levels of *AmCHR*, *AmCHI*, *AmIFS* and *AmI3'H* in leaves were decreased by about 2.00-, 2.35- ,3.55-,5.25 times, respectively. The expression levels of *AmCHR*, *AmCHI*, *AmIFS* and *AmI3'H* in roots were increased by about 4.76-,2.71-,8.01- and 5.57 times, respectively. The expression level of *AmCHS* (Fig. 4d) in the stem was the highest, which was increased by about 1.86 times compared with the control. The expression level of *AmCHS* in the leaves and roots decreased by about 2.51- and 3.04 times respectively, while the expression level of *AmIOMT* in the leaves was the highest, which was increased by about 6.69-,3.93- and 1.59 times respectively compared with the control.

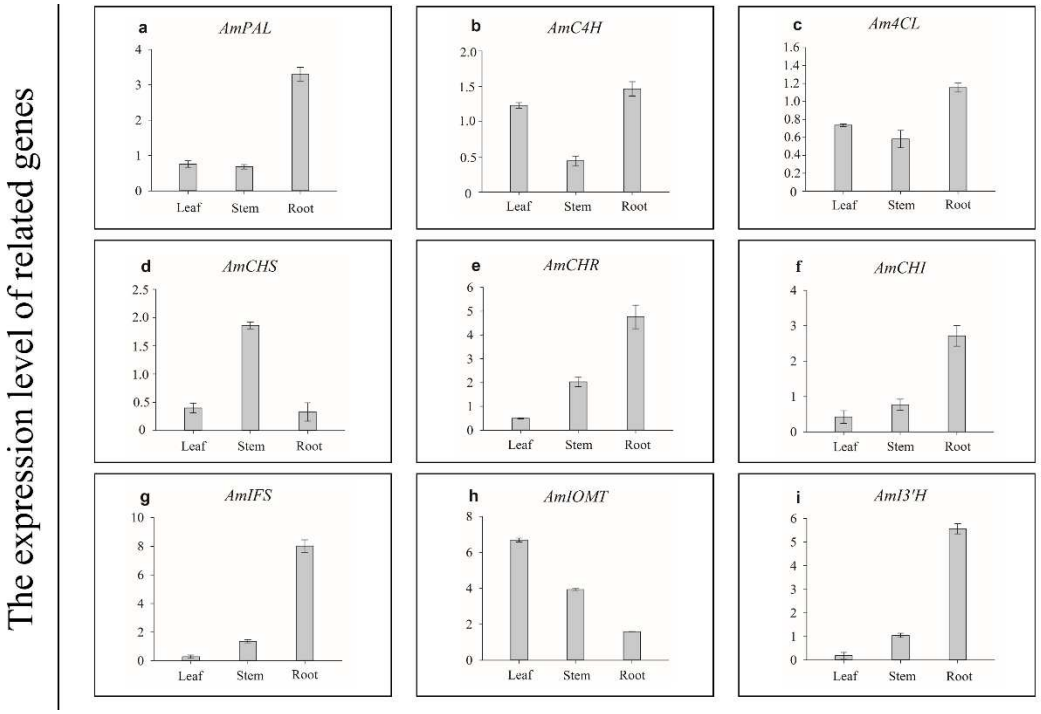


Fig.4 Effect of drought stress on the expression of genes related to the calycosin and CG biosynthetic pathways in different organs of *A. mongholicus*. (a) *AmPAL*; (b) *AmC4H*; (c) *Am4CL*; (d) *AmCHS*; (e) *AmCHR*; (f) *AmCHI*;

198 (g) *AmIFS*; (h) *AmIOMT*; (i) *AmI3'H*. The height of each bar and the error bars indicate the means and standard
199 deviation, respectively, from three independent measurements.

200 **The Expression Level of Related Genes in Different Organs of *A. mongholicus* Under Rewater**

201 Fig. 5 showed the expression of *AmIOMT*, *AmIOMT*, *AmCHR*, *AmIFS*, *AmI3'H*, *AmPAL, AmC4H*,
202 *Am4CL* and *AmCHI* in roots, stems, and leaves after rewater, these genes showed an increased
203 trend in leaves, except *AmCHR*, *AmCHI* and *AmI3'H*. PAL and C4H, catalytic synthesis of cinnamic
204 acid and 4-coumaric acid respectively, in the CG pathway, was downward expression levels in
205 stems of *A. mongholicus* with rewater treatment, just as the trends of cinnamic acid and 4-coumaric
206 acid. *AmPAL*, *AmC4H*, *Am4CL*, *AmCHS*, *AmIFS*, *AmIOMT* all exhibited the highest expression
207 levels in leaves compared with the roots and stems, increasing by about 3.17-, 1.34-, 4.98-, 6.31-,
208 3.57- and 2.42 times respectively. *AmCHS* expression level in the roots was decreased by about
209 2.06 times. The expression of *AmPAL*, *AmC4H*, *Am4CL*, *AmIFS* and *AmIOMT* in the root was
210 higher than that in the stems, these genes increased about 1.24-,1.05-,2.36-,1.02- and 1.14 times
211 respectively in the root. The expression levels of *AmPAL*, *AmC4H*, *AmIFS* and *AmIOMT* in the
212 stem decreased by about 1.42-, 1.82-, 1.18- and 1.35 times respectively. The expression levels of
213 *AmCHR*, *AmCHI* and *AmI3'H* were the highest in the stems compared with the roots and leaves,
214 meanwhile the expression levels of these genes increased by about 4.67-,4.36- and 3.11 times
215 respectively. The expression levels of *AmCHR*, *AmCHI* and *AmI3'H* in the leaves increased by
216 about 3.04-1.29- and 1.33 times respectively, while the expression levels of these genes in the roots
217 decreased by about 4.95-, 1.03- and 1.33- fold respectively.

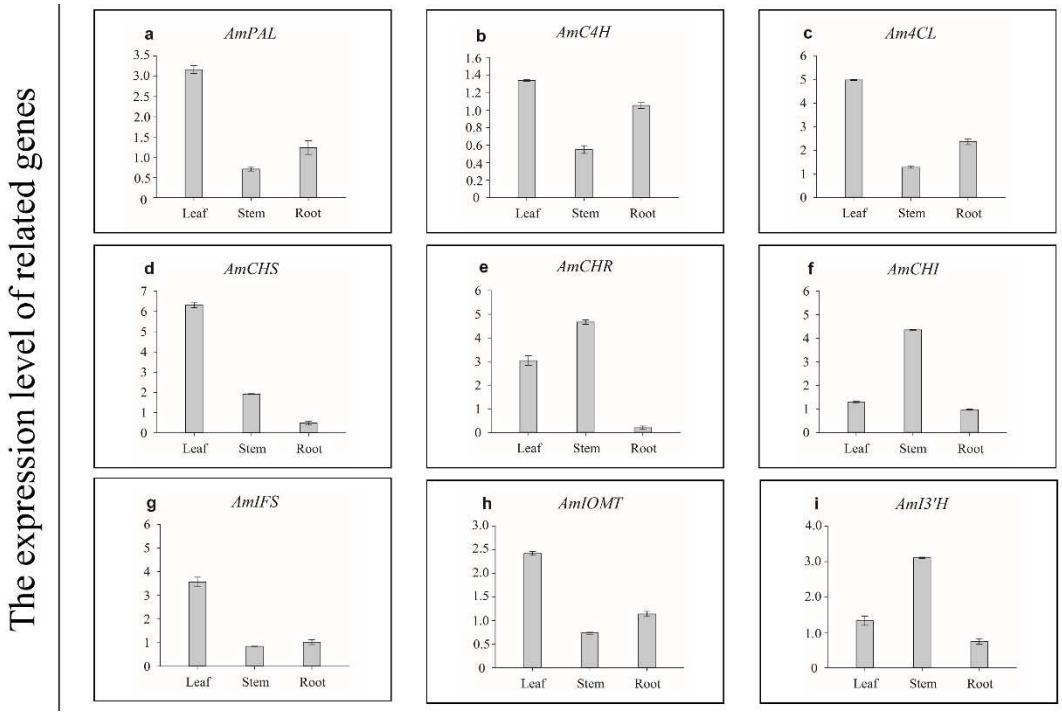


Fig.5 Under rewater, expression levels in the leaves, stems, roots of genes related to the calycosin and CG biosynthetic pathways. (a) *AmPAL*; (b) *AmC4H*; (c) *Am4CL*; (d) *AmCHS*; (e) *AmCHR*; (f) *AmCHI*; (g) *AmIFS*; (h) *AmIOMT*; (i) *AmI3'H*. The height of each bar and the error bars indicate the means and standard deviation, respectively, from three independent.

Accumulation of Metabolites Related to CG Synthesis in Different Organs Under Drought Stress

The content of compounds on the CG synthesis pathway was determined in different organs of *A. mongholicus* by UHPLC-MS /MS under drought stress, as shown in Fig. 6. All target compounds, in addition to calycosin, exhibited enhanced accumulation in response to drought stress in roots of *A. mongholicus*. The content of cinnamic acid, liquiritigenin, daidzein and CG in roots were 0.1389 μg /g DW (dry weight), 0.2198 μg /g DW and 0.0643 μg /g DW respectively, which were 2.86-,12.93-, 34.63 times than those of the control. L-phenylalanine and 4-coumaric acid were

accumulated significantly in leaves after treatment. The contents of L-phenylalanine and 4-coumaric acid were 76.3323 μg /g DW and 0.3555 μg /g DW, respectively, which were 5.90 and 1.99 times higher than the control. Drought induced accumulation of formononetin and calycosin in stems were 0.2632 and 0.6039 μg /g DW respectively, which was 6.79- and 7.19 times over the levels in control plants. The content of CG in roots was15.8522 μg /g DW, increased remarkably compared with the control.

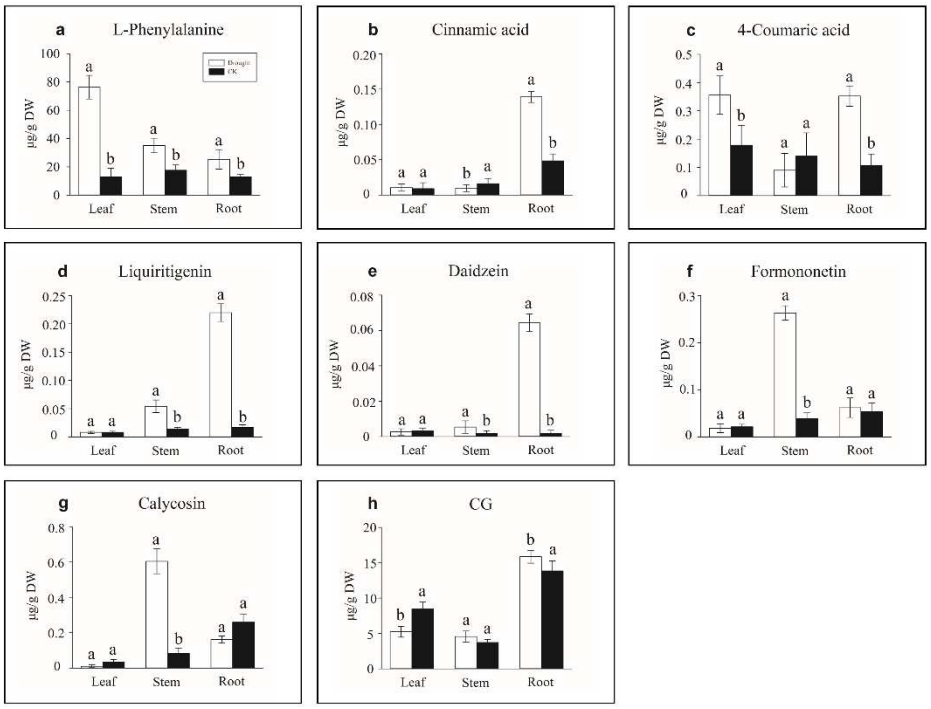


Fig.6 Effect of drought stress on the content of metabolites related to calycosin-7-*O*-*β*-D-glucoside biosynthetic pathways in different organs of *A. mongholicus*: (a) L-Phenylalanine; (b) Cinnamic acid;(c) 4-Coumaric acid; (d) Liquiritigenin; (e) Daidzein; (f) Formononetin; (g) Calycosin; (h) CG. Data represent the mean values ± SE. Different letters indicate significant differences by Tukey's test ($P < 0.05$).

Accumulation of Metabolites Related to CG Synthesis in Different Organs Under Rewater

The contents of related compounds in different organs were detected under rewater. As shown

in Fig. 7, liquiritigenin, formononetin and calycosin exhibited increased accumulation in response to rewater elicitation in roots, stems and leaves of *A. mongholicus*. In contrast, the accumulation of cinnamic acid in stems and roots was decreased. Rewater induced accumulation of daidzein in stems and the opposite trend appeared in leaves. As shown in Fig. 7d and Fig. 7e, the contents of liquiritigenin and daidzein in stems were 0.0748 μg /g DW and 3.4387 μg /g DW, which were 4.38- and 1.78 times higher than the control. More cinnamic acid, formononetin, calycosin and CG were accumulated in roots than stems and leaves after rewater. The contents of formononetin and calycosin in roots were significantly increased by 0.81- and 0.78-time compared with the control. In contrast, the content of cinnamic acid in roots was significantly decreased by 1.41 times compared with the control. However, no change in content of CG in roots is achieved compared with the control.

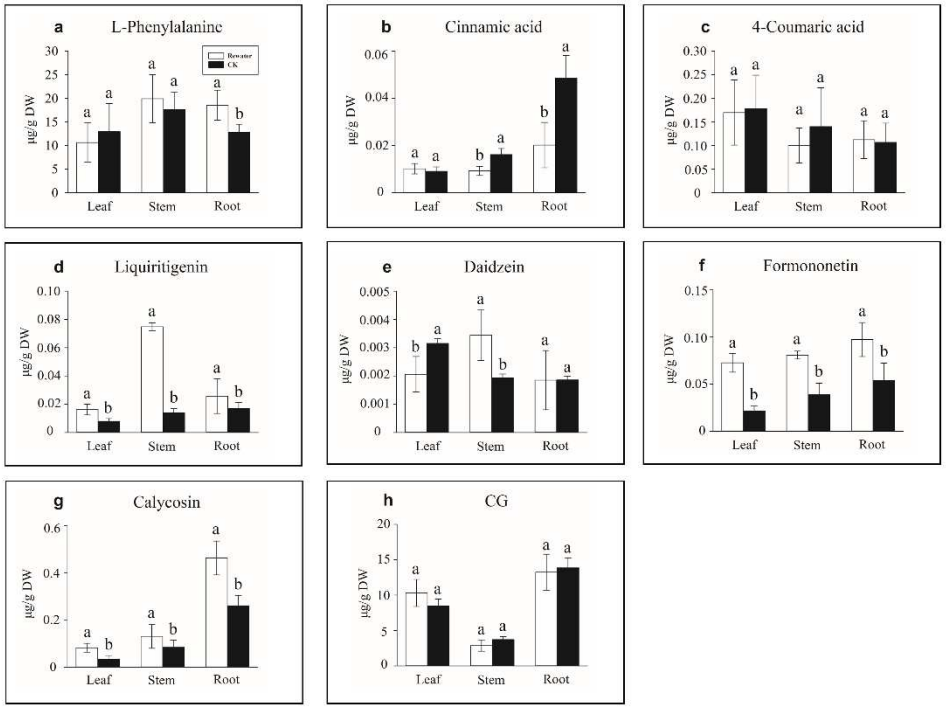
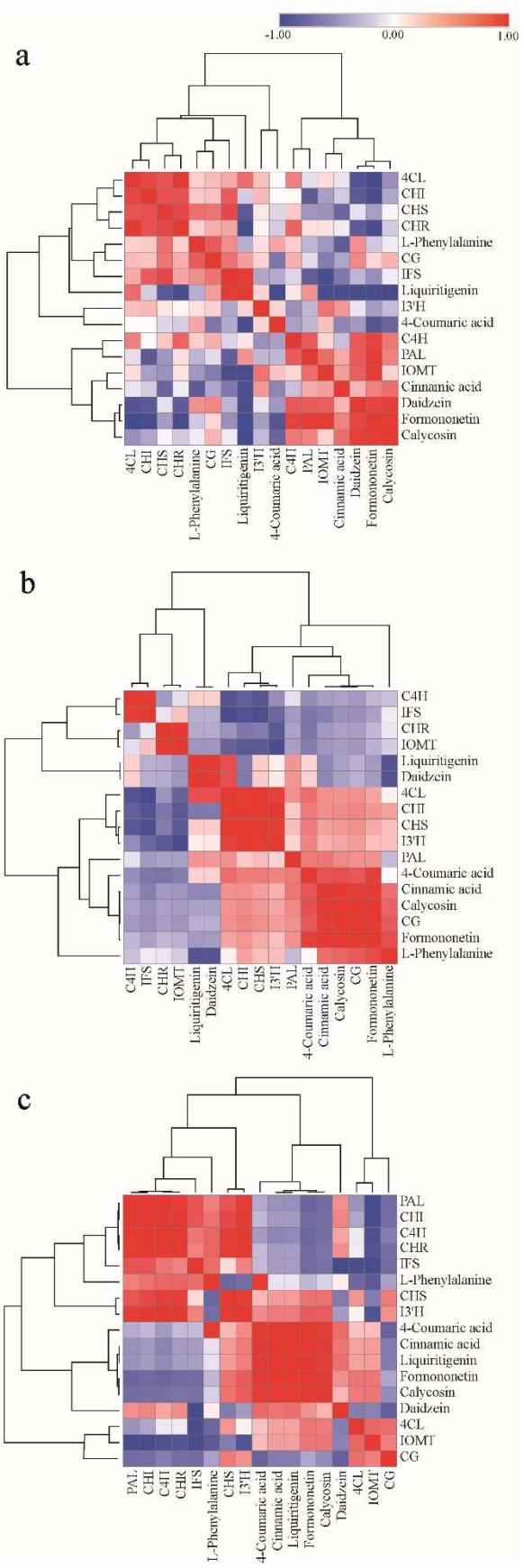


Fig.7 Effect of rewater on the content of metabolites related to calycosin-7-*O*-*β*-D-glucoside biosynthetic pathways in different organs of *A. mongholicus*: (a) L-Phenylalanine;(b) Cinnamic acid;(c) 4-Coumaricacid; (d) Liquiritigenin;(e) Daidzein;(f) Formononetin;(g) Calycosin;(h) CG. Data represent the mean values ± SE. Different letters indicate significant differences by Tukey's test ($P < 0.05$).

Correlation analysis of target metabolites and related enzyme genes in CG pathway

During this process, most of the metabolites and key enzyme genes related to the CG pathway changed under water stress. In order to clarify whether there was a correlation between these variations, correlation analysis of targeted metabolites and related genes in the leaves, stems and roots were demonstrated. As shown in Fig. 8, *AmIOMT* in leaves showed a strong negative correlation with the content of liquiritigenin, but have a strong positive correlation with formononetin. Interestingly, *AmIFS* also exhibited a strong positive correlation with the accumulation of liquiritigenin (Fig. 8a). In the stems, *AmPAL* was moderately correlated with the accumulation of cinnamic acid, while *Am4CL* was strongly correlated with the accumulation of liquiritigenin and daidzein (Fig. 8b). In roots, *AmC4H* was moderately correlated with L-phenylalanine content. *AmIFS* was negatively correlated with the accumulation of daidzein. a strong correlation between *AmI3'H* and the accumulation of calycosin existed (Fig. 8c).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a
4CL
CHI
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CHR
L-Phenylalanine
CG
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Liquiritigenin
I3'H
4-Coumaric acid
C4H
PAL
IOMT
Cinnamic acid
Daidzein
Formononetin
Calycosin
b
C4H
IFS
CHR
IOMT
Liquiritigenin
Daidzein
4CL
CHI
CHS
I3'H
PAL
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Cinnamic acid
Calycosin
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I3'H
4-Coumaric acid
Cinnamic acid
Liquiritigenin
Formononetin
Calycosin
Daidzein
4CL
IOMT
CG

Fig. 8 Correlation map of target metabolites and relative genes in (A) Leaves (B) Stems (C) Roots of *A. mongholicus* under drought stress. Each square indicates r (Pearson's correlation coefficient values for pairs of isoflavones or relative genes values). The red color represents a positive ($0 < r < 1$) correlation, and the green color represents a negative ($-1 < r < 0$) correlation.

Discussion

In addition to causing material transfer between the shoot and root, drought also increased the root-shoot ratio. Proline is the main organic osmotic agent, which plays an important role in the response of plants to abiotic stress, such as reducing the osmotic potential of cells to promote plant retention or absorption of water[34]. Previous studies have shown that Astragalus can increase proline levels under water deficit stress[35]. In this study, the content of proline increased significantly with time under drought-stress treatment, reached the peak on day 15, and it was particularly higher in stems than in leaves. Drought stress led to lipid peroxidation, the production of MDA, and ultimately generated cell damage and plant death[36]. We observed that drought stress significantly increased the MDA content, reaching a peak on day 12, and the MDA content in roots was higher than that in leaves and stems, indicating that the roots suffered more severe lipid peroxidation.

Moderate drought stress can promote the accumulation of CG [29], however the change in the accumulation of intermediate metabolites is not clear. We found that liquiritigenin was accumulated significantly in roots and stems of *A. mongholicus* under drought stress, and similar results were also obtained in *Glycyrrhiza uralensis* [37]. Previous studies have shown that the contents of daidzein, formononetin in soybean roots were increased under water deficit[38], which is consistent

293 with our results (Fig. 6). However, the accumulation of them is decreased, in soybean roots under
294 salt stress, which may be due to the fact that soybean mainly formed 24 dihydroxy b-ring flavonoids
295 instead of 40 1-hydroxy b-ring flavonoids in response to salt stress[39]. Interestingly, the
296 accumulation of daidzein and formononetin in soybean leaves was decreased under high
297 temperature [6]. The content of them in soybean root was increased after low temperature
298 treatment[40]. After salt stress, the contents of calycosin and CG in roots, stems and leaves of *A.*
299 *mongholicus* were decreased [33]. Pan's study found that CG content in *A. mongholicus* root
300 decreased during cold treatment, while no significant change in calycosin was observed [41]. In
301 general, flavonoids have antioxidant functions when higher plants are challenged by a series of
302 environmental pressures [12]. At present, there is a view that the increased calycosin in roots may
303 arise from leaves and stems. Some studies have shown that metabolic compounds are usually
304 transported from the synthetic site to the accumulation site of plants [42, 43]. In addition, the
305 accumulation of phenylpropanoid is discrepant in various organs of the same plant when the plant
306 grows under stress[44].

307 The biosynthetic pathway of flavonoids is one of the crucial secondary metabolic pathways in
308 plants, and many key genes responding to environmental stress have been studied [45]. Under
309 drought stress, *AmPAL*, *AmC4H* and *Am4CL* showed similar expression patterns, elevated
310 transcription levels in roots indicated that phenols in the roots were stimulated in response to
311 drought and these genes showed similar expression trends in most cases. Previous reports also
312 highlighted the co-expression of these genes under drought conditions [45]. CHS is thought to be

313 the entry point of the flavonoid pathway, where CHI converts chalcone to flavanone compounds, a
314 prerequisite for many other flavonoid compounds [45]. *CHS* gene expression was the highest in
315 the period of strong drought stress [46]. Previous studies found that water deficit can contribute to
316 the expression of *CHS* in leaves and roots of *Scutellaria baicalensis* [47]. The expression of some
317 genes overlaps under drought, salt stress and low temperature stress [48]. Margarita et al. found
318 that the expression of *AmIFS* in the leaves of *Lotus japonicas* was increased under drought
319 treatment [49], while our study showed that the expression level of *AmIFS* in the leaves of *A.*
320 *mongholicus* was decreased under drought treatment but increased significantly in the roots (Fig.
321 4). This may be due to different storage organs of isoflavones, leading to various expression levels
322 of *AmIFS*. The transcription level of *AmPAL*, *AmC4H*, *AmCHI* and *AmIFS* were up-regulated in
323 roots under drought induction, and down-regulated in leaves and stems. This change is similar to
324 the accumulation pattern of the corresponding compounds of these enzymes. For example, the
325 isoflavones of *Lotus japonicus* are mainly stored in leaves [50], while the isoflavones of *A.*
326 *mongholicus* are mainly stored in roots. *AmIOMT* is highly expressed in the context of water
327 deficiency, which has similar results in chickpeas (*Cicer arietinum* L.) [51]. It has been reported
328 that the expression level of *I3'H* was increased significantly in response to drought, low
329 temperature and salt stress [52]. *I3'H* in roots of *A. mongholicus* was highly expressed in response
330 to drought stress (Fig. 4). These results show that the high expression of *IFS* and *I3'H* contributes
331 to the accumulation of CG in roots after drought stress.
332 In this study, drought stress exhibited an obvious induction effect on the accumulation of

isoflavones in *A. mongholicus* (Fig. 6). The expression level of *AmIOMT* in the leaves was the highest under drought stress, while the product of IOMT catalyzed synthesis, formononetin, was accumulated mostly in the stem (Fig. 4, Fig. 6). One possible origin is that significant increased *AmIOMT* expression level in leaves, leading to a large amount of formononetin synthesis in leaves, which is then transported to the stem through the petiole. This is consistent with previous reports. Isoflavones were found in the secretion of phloem of *Astragalus membranaceus* petiole [41]. Another reason is probably that *AmIOMT* interferes or even blocks the process of regulating and synthesizing formononetin during water deficiency. The accumulation of calycosin and CG in leaves was significantly reduced under drought stress compared with the control one, indicating that formononetin might be transported to other organs instead of being transformed into downstream metabolites in leaves[31, 43]. Previous evidence has demonstrated that under drought stress, the accumulation of CG, an active ingredient with medicinal value, in *Astragalus membranaceus* root increased under drought stress [28, 35], which probably results from calycosin, a precursor compound synthesized from CG in stem[41, 53].

Conclusions

Previous studies mainly focused on the influences of abiotic stress on CG content, which is the final metabolite content in the Calycosin-7-*O*-*β*-D-glycoside (CG) synthesis pathway, while the content of all intermediate metabolites in this pathway was measured in this research. Moreover, the regulation mechanism of CG accumulation in different organs under continuous drought conditions was studied. The accumulation of calycosin and CG in roots under drought stress may

be derived from formononetin synthesized in leaves. This study explored the flavonoid synthesis of plants undergoing abiotic stress on the level of metabolites and key enzyme gene expression, which is of great significance for obtaining high-quality *Astragalus membranaceus* in arid or semi-arid area. In traditional Chinese medicine, the root of *Astragalus membranaceus* is used and most of its stems and leaves are discarded. Therefore, the future research will focus on the comprehensive utilization of the non-medicinal parts of astragalus species.

Ethics declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable. The manuscript does not contain any individual person's data.

Competing Interests

The authors declare no competing interests.

Author contribution

Yinghui Chen and Bingzhen Li conducted the experiments. Yinghui Chen carried out the data analysis and drafted the manuscript. Guilin Chen, Shuying Sun and Youla Su provided experimental assistance to Yinghui Chen and Bingzhen Li; Guilin Chen (major parts), Xin Jia revised the manuscript. Guilin Chen with Yinghui Chen designed the experiments and the manuscript. All authors discussed the results and approved the final version of the manuscript.

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Availability of Data and Materials

Not applicable.

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