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POSTHARVEST UV-B IRRADIATION IMPROVES THE ACCUMULATION OF FLAVONOID AND BIOSYNTHETIC GENE EXPRESSIONS IN *SCUTELLARIA BAICALENSIS* ROOT

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Abstract

To examine the effects of postharvest UV-B radiation on the accumulation of flavonoid in *Scutellaria baicalensis* root and the underlying mechanism, the quantitative analysis of the flavonoid contents (baicalin, wogonoside, scutellarin, baicalein and wogonin) was performed using HPLC. The expressions of key enzyme genes PAL, 4CL, CHS, CHI, UBGAT and GUS in the biosynthesis of flavonoid were examined using RT-PCR. The value of flavonoids, especially baicalin, wogonoside and wogonin, displayed a time-dependent increase, and the content of baicalin in the control reached 80.7 mg/g at 12 h during drying process. The content of baicalin in UV-B treatment was increased significantly by 69.67% ($p<0.05$) and 51.32% ($p<0.05$) respectively compared with control at 12 h (109.14 mg/g) and 24 h (97.35 mg/g) and reach the maximum value at 24 h. The key enzymes exhibited different expression patterns during the postharvest drying process. The mRNA levels of key enzyme genes CHS, CHI, GUS and PAL in the control were observed to be stimulated, reaching peak levels at 12 h, and their maximum value reached 3-, 3-, 4-, 5- and 4-fold of that at 0 h respectively. While they exhibited an even more increase in UV-B irradiation treatment compared to the control. Appropriate water deficit in dehydration could significantly promote the levels of flavonoids in *S. baicalensis* roots and suitable concentration of UV-B treatment is an effective approach for promoting the content of flavonoids during the postharvest drying process.

Key words: Traditional Chinese medicine, *Scutellaria baicalensis*, Secondary metabolites, Postharvest, UV-B irradiation, Flavonoids.

Abbreviations: PAL, phenylalanine ammonialyase; 4CL, p-coumaroyl CoA ligase; UBGAT, UDP-glucuronate: baicalein 7-*O*-glucuronosyltransferase; GUS, glucuronidase; CHI, chalcone isomerase; CHS, chalcone synthase.

Introduction

The content of secondary metabolites in medicinal plants is often improved by postharvest treatments. The postharvest increase of secondary metabolites in medicinal plants was also caused by applying abiotic stresses, such as light exposure, temperature and heating (Harbaum-Piayda *et al.,* 2016). Appropriate UV-B radiation may induce photobiological stress, activate the defence response and result in increasing in the content of secondary metabolites (Eichholz *et al.*, 2011). It has been shown that plants can respond to UV-B radiation by starting up defensive mechanism which is the biosynthesis of UV-B absorbing ingredients, such as flavonoids (Koes *et al.*, 2005; Du *et al.*, 2011). For example, the concentrations of the total flavonoids in peel and flesh of apples were increased after postharvest UV-B irradiation (Frohnmeyer & Staiger, 2003; Tang *et al.*, 2011; Wei *et al.*, 2012; Nechet *et al.*, 2015; Harbaum-Piayda *et al.*, 2016; Rybarczyk-Plonska *et al.*, 2016; Formica-Oliveira *et al.*, 2017).

Scutellaria baicalensis Georgi, belongs to the family Scrophulariaceae, referred to in Traditional Chinese medicine (TCM) as "Huang qin", is one of the most renowned herbs in traditional Chinese medicine and has been widely used to treat pneumonia, diarrhea, fever, liver cirrhosis and anxiety (Son *et al.*, 2017; Liu *et al.*, 2017). Flavonoids such as baicalin, wogonoside, wogonin and baicalein have been recognized as the major bioactive compounds in Huang qin and are also important indexes to measure the quality of Huang qin (Zhou *et al.*, 2012; Zhao *et al.*, 2016). Flavonoids in *S. baicalensis* are reported to be an essential ingredient in protecting plants against external stresses such as drought and low temperature. The biosynthesis of flavonoids in root of *S. baicalensis* was increased by UV-B radiation (Marsh *et al.*, 2014). However the underlying mechanism of the improving of flavonoids in root of *S. baicalensis* regulated by UV-B has not been clear. Flavonoids are synthesized with series of key enzymes and speed limiting enzymes, such as phenylalanine ammonialyase, PAL; p-coumaroyl CoA ligase, 4CL; cinnamoyl 4 hydroxylase, C4H; chalcone isomerase, CHI and chalcone synthase CHS, which are parts of phenylpropanoid metabolism (Zhao *et al.*, 2016; Tohge *et al.*, 2017). However, little is known about the effects of UV-B radiation on the changes of flavonoids and corresponding biosynthetic gene expression.

The objectives of the present research were (i) to study the effects of postharvest UV-B radiation on the content of flavonoids (baicalin, wogonoside, scutellarin, baicalein and wogonin) in *S. baicalensis* and (ii) to evaluate the expressions of key enzyme genes PAL, 4CL, CHS, CHI, UBGAT and GUS in the biosynthesis of flavonoids.

Material and Methods

Plant material and UV-B radiation treatment: The fresh roots (three years old) were collected from Laiwu *S. baicalensis* cultivation base, Shandong Province in China in October 2017, which is the best time for collection of *S. baicalensis* in this region. The roots were washed with running water and directly selected to conduct UV-B induction experiments. One hundred and twenty-six roots with uniform size and free of visual blemishes and disease were used in these experiments. The roots were divided evenly into two parts. Among them one part was subjected to natural lighting (as control), while the other one was subjected to UV-B irradiation with the dosages of 5 kJ/m^2 for 4 h at a distance of 30 cm above the roots. Following treatments, the roots in both the control and the treated group were air-dried at room temperature, sampled at adaptation time point (0 d, 0.5 d, 1 d, 3 d, 5 d, 7 d and 15 d after treatment) and respectively separated into 3 groups for determination of moisture content, the content of flavonoids (after heating at 105^{o}C for 5 min and drying to constant weight at 40^{o}C) and analysis of main genes expression (after freezing with liquid nitrogen and storing at -80^{o}C) respectively. The above measurements were repeated 6 times.

Extraction and qualitative analysis of flavonoids compounds: The quantitative analysis of the flavonoid contents of *S. baicalensis* was measured using HPLC. For the analysis of baicalin, wogonin, wogonoside, scutellarin and baicalein and in *S. baicalensis*, the extraction was conducted according to Liu *et al*., (2017). The dried samples (0.5 g each) were ground into power (80 mesh), and extracted with 70% ethanol (45 mL) for 50 min under ultrasonication (250 w, 25 kHz, 60^{o}C). Then, the extract was centrifuged at 10000 r/min for 15 min, and then passed through a membrane filter of 0.45 μm. Agilent 1200 Series liquid chromatograph with a DIKMA Diamonsil^{TM}-C18 column at 25^{o}C was used to analyze the contents of flavonoid in *S. baicalensis*. The detection wavelength was 275 nm, the flow rate was 1 mL·min^{-1} and a sample injection volume was 5 mL. The mobile phase consisted of A (0.2%-methanoic acid-ammonium) and B (acetonitrile), using a gradient of 85%-85% A at 0-5 min, 85%-80% A at 5-10 min, 80%-77% A at 10-15 min, 77%-75% A at 15-17 min, 75%-70% A at 17-30 min, 70%-60% A at 30-35 min, 60%-55% A at 35-40 min and 55%-0% A at 40-55 min. The theoretical plate number was calculated according to the peak of baicalin, which should not be less than 15000. The reference standards were purchased from Chengdu Mansite Pharmacetical CO LTD., Chengdu, PR China.

Analysis of expressions of key enzyme genes: Expressions of key enzyme genes PAL, 4CL, CHS, CHI, UBGAT and GUS in the biosynthesis of flavonoid were examined during the drying process. Total RNA was extracted based on CTAB following the method of Zhang *et al*., (2014). Then RNA was reversely transcribed to generate cDNA using the manufacturer's methods of PrimeScript RT reagent kit. Primers were designed as followed: PAL forward primer 5′-ACTCTTCTTCAAGG ATACTCA-3′; PAL reverse primer 5′-GTGATGTT GTGGTTAAGGAA-3′; 4CL forward primer 5′-GAAG AAGCAGGAGAAGTT-3′; 4CL reverse primer 5′-TGGAATGGCATCAATGAA-3′; CHS forward primer 5′-GATGGATGAGATGAGGAA-3′; CHS reverse primer 5′-TGGAATGGCATCAATGAA-3′; CHI forward primer 5′-CTGCTCCTTGACGATTAG-3′; CHI reverse primer 5′-GCTGTTTGTTCTCTATTACTG-3′; UBGAT forward primer 5′-AGCCAAGGAAGCCATAGTCAAC-3′; UBGAT reverse primer 5′-CCGAAACAAAGGAAGA CGACA-3′; GUS forward primer 5′-AGAGCAGTGT GAAGATAAGC-3′; GUS reverse primer 5′-CATAGTA GGTCCAGGCAAG-3′. Real-time PCR was performed following the methods of SYBR Prem ix EX Taq II kit (Takara, Shiga, Japan): an initial denaturation step of 95°C for 10 min, 95°C for 15 s, and then 40 cycles of 55°C for 30 s, 1 min at 72°C and 95°C for 15 s, followed by a extension step of 60°C for 1 min, 95°C for 15 s, and 50°C for 30 s. Transcript fold-changes were calculated by the $2^{-\Delta\Delta Ct}$ method, and β-Actin was used as internal standard and was amplified with the endogenous actin gene (actin-forward 5′-AGTCCACTGAACCTTATC-3′; actin-reverse 5′-CTCCCTTTGATGTTTGAT-3′) (Livak & Schmittgen, 2001).

Statistical analysis

One-way ANOVA was performed using SPSS software (SPSS Statistics 20.0). Duncan's Multiple Rang Test was used to separate means at $p<0.05$.

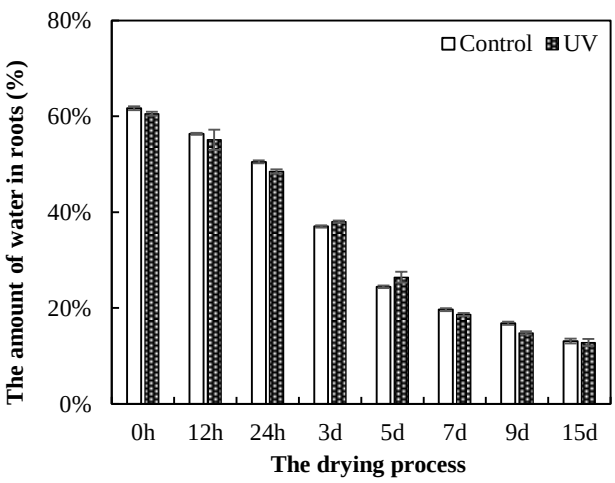

Fig. 1. Changes of water content in roots of *S. baicalensis* during the drying process after UV-B irradiation.

Results

Effects of UV-B irradiation on the amount of water in the roots during drying process: As shown in Fig. 1, the content of water both in the control and in UV-B treated roots depicted significant decrease during the drying process. The changes trend of water content in *S. baicalensis* roots treated with UV-B radiation was investigated and compared with the control in order to find out any significance difference. A significant sharp decline was observed in the amount of water at 24 h drying process,

which was evaluated with18.14% (control, $p<0.05$) and 19.89% (UV-B treatment, $p<0.05$) respectively compared with that at 0 h. While the content of water in roots displayed a slowdown from 24 h to 15 d and reached the lowest value 13.10% (control) and 12.81% (UV-B radiation) on 15 d during the drying process. The water content in the control and in the UV-B treated roots exhibited a significant declining phenomenon during the postharvest drying process, especially within 24 h after drying. Little difference of water content between control and UV-B radiation group was detected in these results.

Effects of UV-B irradiation on the amount of flavonoid in *S. baicalensis* roots during drying process: The flavonoid profile in *S. baicalensis* roots showed a significant increment during drying process both in the control and in UV-B treated roots. As shown in Fig. 2A, the content of baicalin in the control samples displayed a time-dependent increase, increasing rapidly and reaching 80.7 mg/g at 12 h during drying process, which was 26.5% higher than that at 0 h. The content of baicalin in UV-B treatment samples was increased significantly by 69.67% ($p<0.05$) and 51.32% ($p<0.05$) compared with control at 12 h (109.14 mg/g) and 24 h (97.35 mg/g) respectively during drying process and reach the maximum value at 24 h during drying process. The contour of wogonoside and wogonin displayed a similar trend to that observed with baicalin. In comparison with that at 0 h, the content of wogonoside in the control samples was observed to be increased by 25.26% ($p<0.05$) at 12 h and by 31.28% ($p<0.05$) at 24 h during drying process. The UV-B treatment resulted in a significant increase in the accumulation of wogonoside by 24.65% ($p<0.05$) on 15 d compared to the control (Fig. 2B). As displayed in Fig. 2C, the content of wogonin was increased and reached 0.65 mg/g at 24 h, 0.78 mg/g at 7 d and 0.81 mg/g at 15 d, which were evaluated as 58.18% (24 h, $p<0.05$), 88.76% (7 d, $p<0.05$) and 95.67% (15 d, $p<0.05$) respectively, higher than that at 0 h in the control. The UV-B treatment was also recorded to be significantly enhanced the content of wogonin, which was 72.29% ($p<0.05$) at 24 h and 83.05% ($p<0.05$) on 15 d higher than that of control. As shown in Fig. 2D, significant reduction in the concentration of baicalein was recorded, which was evaluated to be 47.03% ($p<0.05$) at 12 h, 40.56 ($p<0.05$) on 5 d and 3.67 ($p>0.05$) on 15 d compared to that at 0 h in the control during the drying process. The concentration of baicalein in UV-B treatment reached 1.41 mg/g, reaching a maximum value on 15 d during drying process, while the content in the control was 1.21 mg/g. The content of scutellarin reached peak level at 12 h, which was recorded to be 2.39 mg/g and was found as 41.17% ($p<0.05$) higher than that in the control. The UV-B treatment significantly enhanced the content of scutellarin, which was recorded as 28.57% ($p<0.05$) at 24 h and 53.65% ($p<0.05$) on 5 d higher than of control (Fig. 2E).

Effects of UV-B irradiation on the expression of key enzyme gene in the synthesis of flavonoid during drying process: In order to evaluate the mechanism of drying-enhanced and UV-B-enhanced the content of flavonoid, the effect of UV-B irradiation on expressions of key enzyme gene in the synthesis of flavonoid was determined. As shown in Fig. 3, the key enzyme exhibited different expression patterns during the postharvest drying process. In the control and UV-B treated roots, expressions of key enzyme gene in the synthesis of flavonoid showed a sharp increase in the early phase (from 0 h to 3 d during drying process) and a significant downward trend in the late stages (from 3 d to 15 d during drying process) was observed. During the postharvest drying process, the mRNA levels of key enzyme genes CHS, CHI, GUS and PAL in the control group were observed to be stimulated, reaching peak levels at 12 h, and their maximum value reached 3-, 3-, 4-, 5- and 4-fold of that at 0 h respectively. The transcriptional level of these key enzymes exhibited an even more increase in UV-B irradiation treatment comparison to the control. Comparing to the control a significant increase in the expression of key enzyme genes PAL, 4CL, CHS, CHI, UBGAT, and GUS by 92.94% ($p<0.05$), 52.32% ($p<0.05$), 36.77% ($p<0.05$), 28.25% ($p<0.05$), 33.88% ($p<0.05$) and 59.36% ($p<0.05$) at 12 h respectively was recorded in the UV-B treatment.

Discussion

The postharvest drying process is actually a resistance to drought stress physiological process for freshly collected roots. For this reason, these roots would activate the anti-drought mechanism and thus would upgrade levels of bioactive components during the early phase of postharvest drying process. Based on the above results, the value of baicalin, wogonoside and wogonin showed an inverse V-shape, *i.e.*, upgraded at first and then degraded during the entire drying process, which was agreed with that reported by Zhang *et al*., (2010). The results revealed that flavonoids play an important role in the possession of anti-drying physiological mechanism in *S. baicalensis* roots. On the other hand, UV-B treatment exhibited obvious effects on the flavonoids in *S. baicalensis* roots during postharvest drying process in this study. UV-B-absorbing compounds, such as flavonoids, could be produced in plants in order to protect from UV-B radiation damage (Tegelberg *et al.*, 2002; Takshak & Agrawal, 2014; Zhang *et al.*, 2017). UV-B irradiation of 5 kJ/m^2 promoted the accumulation of flavonoids in *S. baicalensis* roots, which was agreed with that reported by Liu *et al*., (2011). UV-B measures have been found to improve the content of active substances in *S. baicalensis* roots during cultivation (Harbaum-Piayda *et al*., 2016). Comparing to the control, it is concluded that suitable concentration of UV-B radiation is an effective approach responsible for promoting the biosynthesis of flavonoids during the postharvest drying process.

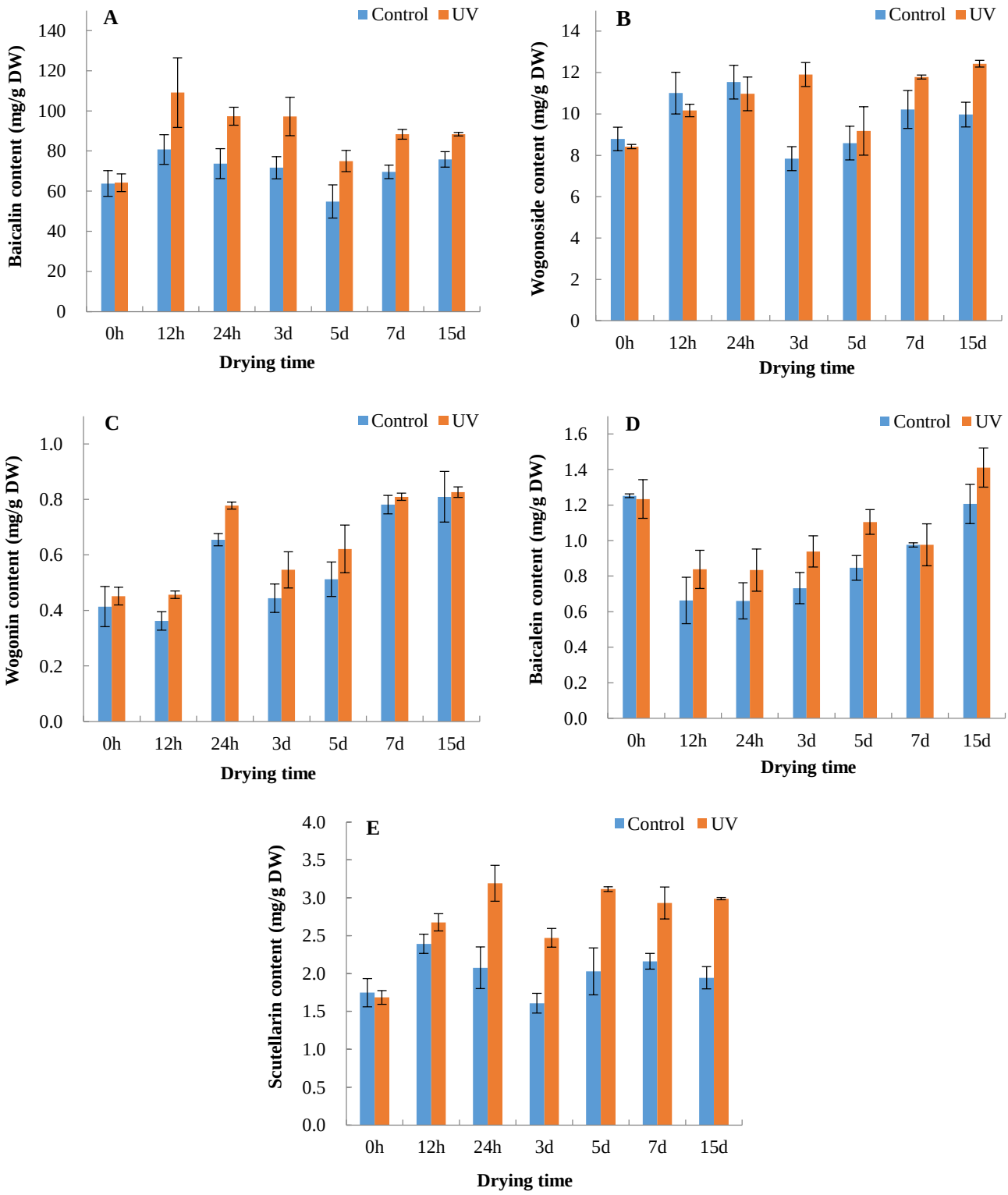

Fig. 2. Content of baicalin (A), wogonoside (B), wogonin (C), baicalein (D) and scutellarin (E) in the roots of *S. baicalensis* during the drying process after UV-B irradiation.

Expressions of key enzyme gene in the synthesis of flavonoid under the control and UV-B radiation treatment was obtained by our study. It provided a possibility to reveal the relationship between enzymatic reaction and the dynamic changes of flavonoid at transcriptional level. In a previous study, the stimulating expression of genes such as PAL, 4CL and CHS in parsley (*Petroselinum crispum* Mill.) were found to reach a higher expression after exposure to UV-B for 5 min and maintained for 24 h (Kanazawa *et al.*, 2012). Another report showed the significance of PAL in flavone biosynthesis in *S. baicalensis* (Eichholz *et al.*, 2011). In this study, UV-B significantly increased the transcriptional level of gene involved in flavonoids biosynthesis pathway. Further experiments were needed to elucidate the underlying mechanism of transcriptional regulation during the postharvest drying process.

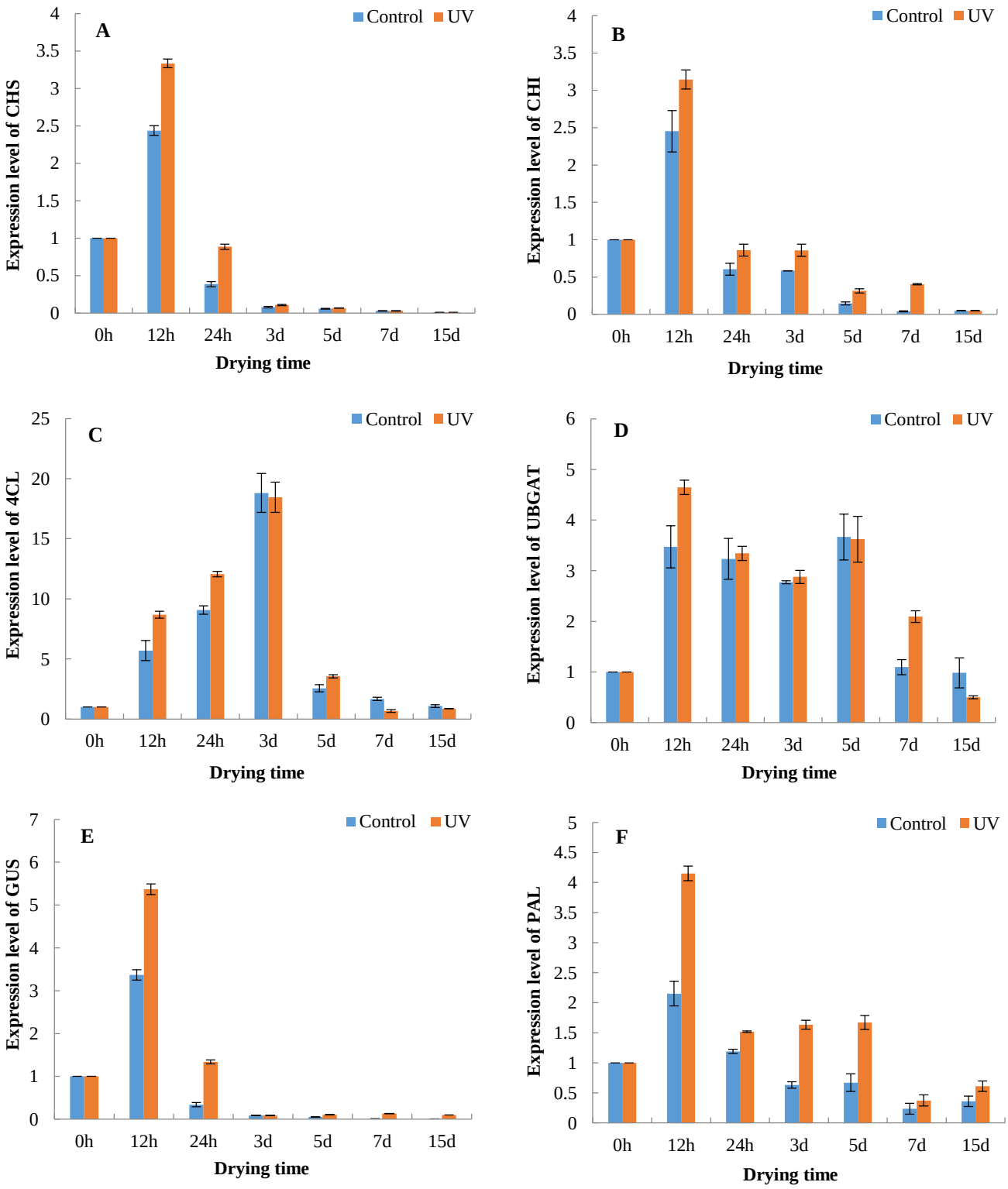

Fig. 3. Expression of key gene enzymes CHS (A), CHI (B), 4CL (C), UBGAT (D), GUS (E), PAL (F) in the biosynthesis of flavonoid during the drying process after UV-B irradiation.

In this study, the dynamic changes of water content, flavonoids content and the expression of key enzyme gene in the synthesis pathway of flavonoids in *S. baicalensis* roots during postharvest drying process were investigated. Based on these findings, this strategy of suitable dose of UV-B radiation could provide a feasible means for improving the content of active ingredient of *Scutellaria baicalensis*.

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