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SHORT COMMUNICATION

Light stress suppresses the accumulation of epimedins A, B, C, and icariin in *Epimedium*, a traditional medicinal plant

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Abstract *Epimedium* is well-known in China and East Asia due to high content of flavonoid derivatives, including icariin, epimedin A, epimedin B, and epimedin C, hereafter designated as bioactive components, which have been extensively utilized to cure many diseases. So far, the molecular mechanism of the bioactive components biosynthesis remains unclear. In the present study, the effect of light stress (24 h illumination) on the accumulation of bioactive components and the expression of flavonoid genes in *Epimedium* was investigated. Under light stress, the structural genes *CHS1*, *CHI1*, *F3H*, *FLS*, *DFR1*, *DFR2*, and *ANS* were remarkably up-regulated while *CHS2* and *F3′H* were significantly down-regulated. For transcription factors, the expression of *Epimedium MYB7* and *TT8* were increased while *Epimedium GL3*, *MYBF*, and *TTG1* expression were depressed. Additionally, the content of bioactive components was significantly decreased under light stress. Our results suggested that the decrease of bioactive compounds may be attributed to transcripts of late genes (*DFRs* and *ANS*) increased to a higher level than that of early genes (*FLS* and *CHS1*).

Keywords Bioactive components · *Epimedium* · Flavonoid biosynthesis · Light stress

Abbreviations

ANS	Anthocyanin synthase
CHI	Chalcone isomerase
CHS	Chalcone synthase
DFR	Dihydroflavonol 4-reductase
F3′H	Flavanone 3′ hydroxylase
F3H	Flavanone 3 hydroxylase
FLS	Flavonol synthase
HPLC	High performance liquid chromatography
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time PCR

Introduction

Epimedium, named as *Yin Yang Huo* in Chinese, has been extensively utilized in China and East Asia because of its health-promoting prenyl-flavonoids components (Ma et al. 2011), such as icariin, epimedin A, epimedin B, and epimedin C, hereafter designated as bioactive components. *Epimedium* extracts are also well known to nourish the kidney, reinforce the Yang, regulate bone remodeling (Ming et al. 2013), promote sexual performance, cure cardiovascular diseases, possess anti-cancer (Tong et al. 2011) and anti-aging benefits (Cai et al. 2011). In the last decades, scientists focused on identifying the phenolic components (Zhao et al. 2008; Zhang et al. 2008; Islam et al. 2008) in *Epimedium* plants. However, little is known about the biosynthesis of prenyl-flavonoids in *Epimedium*

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(Fig. 1a). In addition, the wild resource of *Epimedium* is endangered because of commercial over-exploitation. Therefore, effective methods, including phytohormones or light treatments, to improve the content of bioactive components in *Epimedium* are alternative (Zeng et al. 2013).

So far, flavonoid biosynthesis has been considered as the well-dissected secondary metabolic pathway (Dixon and Steele 1999). As shown in Fig. 1a, structural genes involved in flavonoid biosynthesis have been isolated and characterized in *Arabidopsis* and other species. Also, these genes have been isolated and characterized in *Epimedium* species (Zeng et al. 2010, 2013, submitted). Many transcription factors, including bHLH, R2R3-MYB, and WD-repeat forming a BMW tricomplex to regulate anthocyanin (GL3/PAP1/TTG1) and proanthocyanin (TT8/TT2/TTG1) biosynthesis, are well studied in *Arabidopsis* (Gonzalez et al. 2008; Baudry et al. 2004). In addition, a flavonol-specific regulator AtMYB12, upregulating *AtCHS* and *AtFLS*, was isolated and characterized (Mehrtens et al. 2005). Although the biosynthetic and regulatory genes in model species such as *Arabidopsis*, tomato, and grape have been well-dissected, those in medicinal plants such as *Epimedium* remain unclear. So far, thirteen *Epimedium MYB* members have been isolated and characterized, among which *Epimedium MYB7* and *MYB9* are homologous to *AtTT2* and *VvMYB5b*, respectively (Huang et al. 2012). Furthermore, transgenic tobacco plant over-expressing *MYB9* accumulate higher level of anthocyanin in flowers than in control (Huang et al. 2012).

In the present study, gene expression detected by real-time PCR and the content of bioactive compound revealed by HPLC in leaves responding to light stress were investigated. Finally, the coordinated relationship of gene expression and phytochemical accumulation is discussed.

Materials and methods

Plant materials and light stress treatment

E. pubescens plants were cultivated in Wuhan Botanical Garden, Chinese Academy of Science, P. R. China before growing to florescence stage. *E. pubescens* plants with approximate 50 % inflorescences flowering were transferred to hydroponic culture and used for light stress treatment. For the light stress treatment, the *E. pubescens* plants were lighted by incandescent lamp at 63 μmol/m^2/s light intensity at room temperature in a light bin and divided into two sets. One set, denoted as control (CT), was lighted under a normal day/night (8 h/16 h) photoperiod. Another set, denoted as light stress (LS), was continuously lighted for 24 h. After treated for 1 day, leathery mature leaves were harvested for investigating gene expression and bioactive components content. Both gene expression and bioactive components content analyses were investigated in three biological specimens and data were presented in a mean value.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Leaves were harvested and powdered in liquid nitrogen. RNA isolation was performed using TRIzol kit (Invitrogen, USA) following the manufacturer's instructions. For qRT-PCR, total RNA was reverse-transcribed with a PrimeScript RT Reagent Kit with gDNA Eraser (DDR047, TaKaRa, Japan), which digested the residual DNA in the RNA samples and reverse-transcribed in a one-step process. Gene transcripts were amplified with a SYBR Premix Ex TaqTM II (DDR081S, TaKaRa, Japan) and detected by an ABI 7500 Real-Time PCR system. The PCR program was as followed: stage 1: sufficient denaturation at 95 °C for 30 s; stage 2: PCR reaction with 40 cycles at 95 °C for 5 s and 60 °C for 34 s; and stage 3: dissociation at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The qRT-PCR experiments were performed in triplicate. The expression level of *Epimedium actin* (*EsActin*) was used to standardize the RNA sample for each qRT-PCR. The expression level of the genes relative to *EsActin* was presented as a fold change. The primers for the qRT-PCR are listed in Table 1.

Bioactive components determination

To analyze bioactive components content, about 50 mg of dry leaves were powdered with liquid nitrogen, soaked in 5 mL of 70 % ethanol, and ultrasonicated for 30 min. The extract was filtered through 0.45 μm poly filters for high performance liquid chromatography (HPLC) analysis. The HPLC analysis at a 272 nm wavelength was carried out using an Agilent Technologies Series 1100 (Agilent Technologies, Palo Alto, CA, USA) at a flow rate of 1.0 mL/min. The chromatographic column used was a Zorbax SB-C18 (250 × 4.6 mm I.D., 5 μm; Agilent Technologies, Palo Alto, CA, USA) operated at a constant temperature of 25 °C. The detailed HPLC program was performed as previous study (Xu et al. 2013). Epimedin A, epimedin B, epimedin C, and icariin standards were purchased from the ChromaDex Company (Santa Ana, USA). Data analysis was performed using the Agilent ChemStation software, version A.10.02.

Results and discussion

Previous studies demonstrate that transcription factors homologous to Arabidopsis *AtTT8*, *AtGL3*, *AtTTG1* are

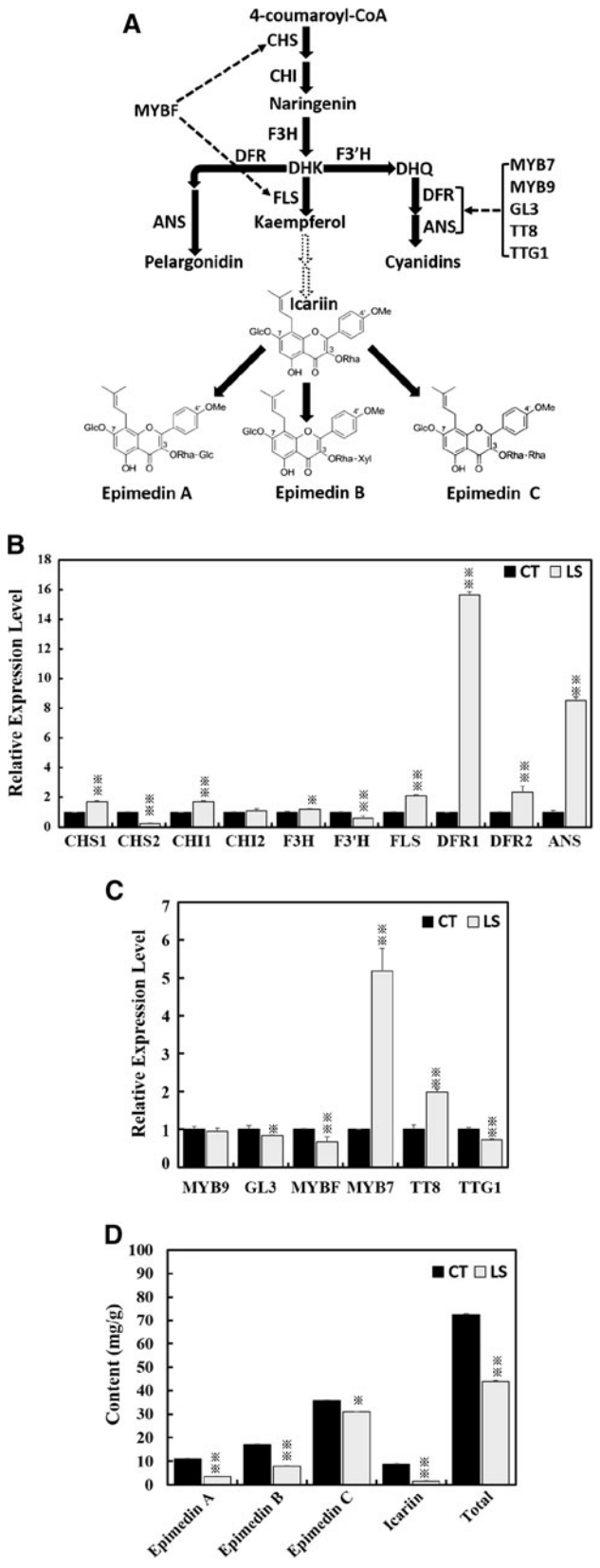

Fig. 1 Biosynthesis of bioactive components in *Epimedium*. **a** Simplified and predicted biosynthetic pathway of the bioactive components of *Epimedium.* Enzyme abbreviations: *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3 hydroxylase, *FLS* flavonol synthase, *F3′H* flavanone 3′ hydroxylase, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanin synthase, *ANR* anthocyanidin reductase. *DHQ* dihydroquercetin, *DHK* dihydrokaempferol. The *dotted arrow* indicate a predicted step committed by enzyme(s) while *black arrows* indicate a step committed by a known enzyme. The *dashed arrows* indicated that the transcription factor(s) putatively regulate corresponding structural genes. Effects of light stress on flavonoid structural genes (**b**) and regulatory genes (**c**) in leaves. Mean ± SD determined from three independent samples are shown. *Double stars* and *single star* respectively indicate significant differences in the amount of gene transcripts at the level of $P < 0.01$ and $P < 0.05$, calculated by Duncan statistical analysis, when compared to CT samples. **d** Light stress impairs the accumulation of bioactive components in leaves. Mean ± SD determined from three independent samples are shown. *Double stars* and *single star* respectively indicate significant differences in the content of bioactive components at the level of $P < 0.01$ and $P < 0.05$, calculated by Duncan statistical analysis, when compared to CT samples

involved in anthocyanin biosynthesis in other plants (Spelt et al. 2000; deVetten et al. 1997; Chiu and Li 2012). Furthermore, both tomato *SlMYB12* (Ballester et al. 2010; Adato et al. 2009) and grape *VvMYBF1* (Czemmel et al. 2009) conserve with Arabidopsis *AtMYB12* in regulating flavonol biosynthesis. These studies showed that transcription factors mentioned above are functionally conserved among plants. In this study, several EST fragments, representing *Epimedium MYBF*, *TT8*, *GL3*, and *TTG1* homologous to Arabidopsis *AtMYB12*, *AtTT8*, *AtGL3*, and *AtTTG1*, were retrieved from the *Epimedium* EST database (Zeng et al. 2010). In addition, flavonoid structural genes had been isolated and characterized in *Epimedium* (Zeng et al. submitted). Together, all structural genes and several transcription factors involved in flavonoid biosynthesis were used to evaluate their effects on the accumulation of bioactive components.

To investigate the effect of light stress on the gene expression of structural genes and regulatory genes involved in *Epimedium* flavonoid biosynthesis, the transcripts of these genes were evaluated by real-time PCR. As presented in Fig. 1b, *CHS1*, *CHI1*, *F3H*, *FLS*, *DFR1*, *DFR2*, and *ANS* expression were significantly up-regulated in LS samples when compared to CT samples. *CHS2* and *F3′H* transcripts were remarkably decreased in LS sample when compared to CT sample. *CHI2* transcripts were increased slightly, but not in a significant level. As shown in Fig. 1c, *Epimedium MYBF* was down-regulated in LS samples, suggesting that *CHS1* expression was upregulated by light stress but not *MYBF* homologous to flavonol-specific transcription factor *AtMYB12* regulating the

Table 1 List of primers used in this study

Primer name	Sequence (5′–3′)
CHS1-RT-F	GGAAGTCTGAGGAGGAAG
CHS1-RT-R	CACATGAACACATACACAATC
CHS2-RT-F	GTGACGGTGCTATTGATG
CHS2-RT-R	AGGCTCTTCTGGATGTTC
CHI1-RT-F	GGAAAGGAAAGTCCGCCGAGGAGT
CHI1-RT-R	AAAATGTGGAGCATACAGTGTAAA
CHI2-RT-F	AGGTTTTCGCTATTCTCGGTGTGA
CHI2-RT-R	ACTCTCTGAAGCAAGCAATGGACA
F3H-RT-F	TCGTGACCTACTTCTCATAC
F3H-RT-R	TTGCCTCAGATAAGACCTC
FLS-RT-F	GGATTGGACTTGAACCTAAC
FLS-RT-R	CCTTGAACACTTGAAGACC
F3′H-RT-F	GCTTGGTGAGTGAGTCTG
F3′H-RT-R	TTCTTTGGGATGTGGTAAC
DFR1-RT-F	CTGCTGGAACTGTTGATG
DFR1-RT-R	CTAGTGTTGGTATGATACTGATG
DFR2-RT-F	TCCATCCGTTACTGTCAC
DFR2-RT-R	TTCACCTTCTTCATGTTAGC
ANS-RT-F	ACTGGAAGAAAACAGACTAGAAAC
ANS-RT-R	CAAGAAGAAGACAATACACAAAGA
MYBF-RT-F	CGAAGAGGGGTCATCTCCTAC
MYBF-RT-R	CCTTTGCCACGAATAAGATGT
MYB9-RT-F	CTTCCACTGCTTCAGCTACCACTA
MYB9-RT-R	GCCACCTACCTTCTCCTTCTCTTT
MYB7-RT-F	CAGGTCTTTTTCACTCCACCGCAG
MYB7-RT-R	ACAGCATCCCTTCACAGGATTCCG
TT8-RT-F	TAATGGGGTTTTTGTGGCTGAGTT
TT8-RT-R	TGATTTGGTGTATTGCCCTTTTCA
GL3-RT-F	TATCGGATGGAACAATCAAACGAG
GL3-RT-R	GCAAAGGTAGAAGACAAAGAAGAA
TTG1-RT-F	ATTCGCTTCCCAACTCTACCTGTG
TTG1-RT-R	AATCAATGCTTGTGAATCATCCCC
Actin-RT-F	TACGAACAGGAGCTGGAGACTT
Actin-RT-R	GATGGTCCAGACTCGTCATACTC

expression of *AtCHS* and *AtFLS* (Mehrtens et al. 2005). The transcripts of *Epimedium GL3* and *TTG1* were also decreased under light stress. In contrast, *Epimedium MYB7* and *TT8* were up-regulated significantly. Results indicated that expression of *Epimedium MYB9* was not affected by light stress under present experimental conditions. To investigate the effect of light stress on the accumulation of bioactive compounds in leaves, the content of bioactive compounds was assayed. As shown in Fig. 1d, the content of epimedin A, B, C, and icariin were significantly decreased in LS sample when compared to CT sample.

The bioactive components content was decreased under light stress, which might be partially attributed to light stress promoting flavonoid degradation (Fahlman et al. 2009) and/or altering the metabolic distribution (Davis et al. 2013). Alternatively, the trade-off of the up-regulated transcripts of *DFR1* and *DFR2* competing with *FLS* for the same substrate (dihydrokaempferol) contribute partially to the decreased bioactive components (Fig. 1). In detail, up-regulated *Epimedium TT8* and *MYB7* promote the high expression of late genes such as *DFRs* and *ANS* although the transcripts of *Epimedium TTG1* was decreased. In Arabidopsis and other plants, MYB (PAP1), bHLH (TT8 and GL3), and WD40 (TTG1) transcription factors form tricomplex BMW to regulate the expression of structural genes, including *DFR* and *ANS*, and further modulate anthocyanin and proanthocyanin biosynthesis (Schaart et al. 2012; Gonzalez et al. 2008; Baudry et al. 2004). Additionally, the increased *Epimedium FLS* transcripts might be only attributed to light stress induction rather than *Epimedium* MYBF because *Epimedium MYBF*, the potential positive regulator of *FLS*, is decreased (Fig. 1c). Previous study documents that *F3′H* is possibly regulated dually by WD-dependent (such as BMW model) and WD-independent (such as flavonol-specific regulator At-MYB12) mechanisms, consisting with its function for producing both quercetin-derivative flavonol and cyanidin-derivative anthocyanin (Gonzalez et al. 2008). The decreased *F3′H* transcripts might be attributed to a large extent to the depressed *Epimedium MYBF* and *TTG1* under light stress even though *Epemidium MYB7* and *TT8* were up-regulated (Fig. 1c). These results suggested that metabolites might be introduced to a large extent into pelargonidin branch. Consequently, the increase in higher level of the *DFRs* and *ANS* transcripts than *FLS* transcripts resulted in funneling more metabolites into anthocyanin/proanthocyanin branches rather than the bioactive components branch.

In conclusion, the expression of genes involved in flavonoid biosynthesis and the content of bioactive compounds were investigated under light stress. Our results suggested that the decrease of bioactive compounds may be attributed to transcripts of *DFR*s and *ANS* increased to a higher level than that of *FLS* and *CHS1*, which led to more metabolites introduced into anthocyanin/proanthocyanin branch than bioactive compounds.

Author contribution Shaohua Zeng designed research, wrote this manuscript and did partial experiment. Yilan Liu did the experiments and Ying Wang designed research.

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